Phenotypic profile and Biomarker Expression Analysis of Human Urinary Bladder Cancers

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine

By

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Declaration

This thesis is the result of my own work except where technical assistance and collaboration with colleagues is acknowledged. The material in this thesis has not been presented, either wholly or in part for any other degree or qualification. The work was carried out during my appointment as a research fellow, employed by Leighton Hospital, Crewe, Cheshire and working in the Pathology department at the University of Liverpool. I collected all the patient data. I reviewed the clinical details and collated the data. I analysed and graded the immunohistochemical staining and reviewed the histopathological diagnosis with Professor C. S. Foster. Dr. Andrew Dodson kindly prepared and stained the slides for the immunohistochemistry. Statistical advice was given by Dr. Sasithorn Willmott and Charis Emmett, Leighton Hospital, Crewe, Cheshire.

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Abbreviations

World Health Organisation/ International Society of Urologic Pathologists	WHO/ISUP
Carcinoma in situ	CIS
Papillary Urothelial Neoplasms of Low malignant potential	PUNLMP
Tumour, Lymph nodes and Metastases staging	TNM
Polycyclic aromatic hydrocarbons	PAH
Cyclin dependent kinases	CDK
Alternate open reading frame	ARF
Oestrogen receptor	ESR

Abstract

Phenotypic and Biomarker Expression Analysis of Human Urinary Bladder Cancers

Background & Objective:

Novel biomarkers are required to distinguish potentially aggressive urothelial bladder cancers from those in which interventional management may be delayed. This thesis tested the hypothesis that accurate biomarker staging would help identify different urothelial cancer pathways to provide essential prognostic information in the pathological assessment of these malignancies.

Design & Setting:

A case-control study with comparative statistical evaluation of biomarker expression and clinical progression in a routine surgical pathology laboratory setting to evaluate the aggressive potential of newly-diagnosed bladder neoplasms.

Patients & Methods:

312 bladder biopsy specimens were examined from 131 unselected patients (87 males and 44 females) and comprising 75% cancers, 4% inflammatory, 8% histologically unremarkable and 13% histological normal urothelial tissue as controls. Tissues were stained immunohistochemically for

expression of a panel of 12 biomarkers (Ki-67, p53, Rb, p21, p16, Bcl-X_L, Bcl-2, PTEN, ERα, ERβ, ErbB-2 and Hsp-27). Staining was dichotomised for analysis using non-parametric statistics. Expression data were compared with histological stage.

Results:

Potential biomarkers Ki-67, p53, Bcl-2, Bcl-X_L, ERβ and ErbB-2 were found to be significantly different between different tumour stages. Significant Up-regulation of Ki-67, p53, p16 and PTEN with loss of Bcl-2, Rb and ERβ was seen in non-muscle invasive urothelial cancer. Significant down-regulation of p16, erbB-2 & PTEN was seen with muscle invasive urothelial cancer. Inflammatory urothelium and urothelium with a history of urothelial carcinoma expressed abnormal expression profiles despite being histologically normal.

Conclusions:

Mutated p53 & Rb identify high-risk non-muscle invasive urothelial cancer. ErbB-2 & bcl-2 distinguish neoplasia from benign disease. Hsp-27 is useful in distinguishing microscopic invasion (pT1) from non-invasive (pTa) malignancies. The findings of this study suggest a significant second hit in muscle invasive urothelial carcinoma with loss of Ki-67, ERβ and erbB-2 and Bcl-X_L gain. Phenotypic classification of urothelial cancer could be based on the four-group expression patterns of p53 and Rb. Thereafter, p21, p16, erbB-2, bcl-2 and PTEN would be useful in sub-group analysis.

Chapter 1

Human Urinary bladder Carcinoma:

--Epidemiology, Staging and Risk Factors

Human bladder carcinoma is a debilitating disease that is often underestimated in terms of incidence, morbidity to the patients and mortality. Worldwide, urinary bladder cancer accounts for over 350,000 new cancers each year being the ninth most common cancer; with 90% of these being urothelial carcinoma. The majority (75-85%) of the urothelial carcinomas are non-muscle invasive at first diagnosis. Despite this, there were over 145,000 deaths[1]. The prognosis remains good with non-muscle invasive urothelial carcinoma despite a high recurrence rate (30%-80%). However, up to 45% progress to muscle invasive disease in 5 years[2 3] . Progression at 5years is more likely to occur in patients who had high grade disease at diagnosis or concomitant carcinoma in-situ[4]. Subsequent 5-year survival rates dramatically lower (<50%) despite radical treatment. Therefore efforts to improve outcome are centred on modifying/ eradicating causative factors as well as attempts to improve early diagnosis and prevention of recurrence and progression.

Non-muscle invasive urothelial carcinoma is a chronic disease with a variable behavioural pattern thereby requiring repeated treatment, frequent follow-up making the cost per patient from diagnosis to death the highest of all cancers[5]. Non-muscle invasive urothelial carcinoma is a major drain on the health economic burden and is also likely to affect the quality of life of patients with bladder cancer. Urothelial carcinoma has a short/unknown prodromal period. Therefore screening tests have been sub optimal. A majority of the patients who present with bladder cancer have haematuria (mainly frank). A low minority of patients have incidentally diagnosed

disease with radiological investigations. Hence, most clinicians and public health scientists have turned their attention towards understanding the epidemiological behavior of this disease.

In this chapter, I intend to outline the evidence available so far on global occurrence, incidence and mortality of urinary bladder cancer and discuss the epidemiological behavior of this disease. The World Health Organisation/ International Society of Urologic Pathologists (WHO/ISUP) consensus classification of urothelial lesions of the urinary bladder is also discussed.

1.1.1 Incidence

Epidemiological studies[1] have failed to show any significant patterns with no comparable urothelial carcinoma incidence between the developing countries and the developed world including Japan. Majority of the urinary bladder tumours were in men (3-times). This marked gender difference is explained to a certain extent by smoking habits and occupation. There was a higher incidence in the developed world with the higher proportion reported in Europe and North America[6]. Work place exposure is the given explanation, estimated to be responsible for up to a quarter of newly diagnosed bladder cancers[7]. In the developing world, industrialisation, less stringent health and safety criteria and an increasing number of smokers together with the population boom have resulted in an increasing incidence especially in the BRIC countries of Brazil, Russia, India and China. The consequences of this demographic shift will only become apparent over the next decade or two. However, the methodology in reporting is circumspect with differences/lack of registering new tumours (Africa and Asia) as well as none/ variable coding of low grade tumours (pTa) in the western world making direct comparisons difficult.

In the European Union, bladder cancer incidence varies across the region with highest incidence (>30/100, 000) among men in northern Italy and Spain. The majority of Northern and Eastern Europe have seen lower rates. United Kingdom, France and Germany have intermediate incidence [6] . However, the ageing population presents a major obstacle with studies

showing an increased incidence with age. Shi *et al.* reported an increase in the percentage of female patients with the non-muscle invasive urothelial carcinoma with increasing age; incidence increasing from 1 in 4 in the under 40s to 1 in 2 in the over 60s[8]. Other studies have reported an increased risk of bladder cancer in post-menopausal women[9]. Hormonal levels particularly oestrogen falls with menopause which suggest a protective effect of oestrogen on the bladder; also shown to inhibit the growth and development murine bladder cancer[10].

1.1.2 Mortality

Age standardised mortality rates (worldwide) are 2-10/100,000 males and 0.5-4/100,000 females [11]. There has been less variability in mortality figures worldwide (Figure 1.1). However, the consequence of an increasing urinary bladder cancer incidence in the developing world is not too difficult to fathom. These countries already have an overstretched and underfunded health infrastructure. Diseases such as urinary bladder cancer with a high per patient cost and morbidity can envisage a sharp increase in mortality.

In Europe, mortality rates were relatively stable until the early 90s with reported rates of 7/100,000 in men and 1.5/100,000 and then declined by more than 21% in next ten years. This could be due to stricter industrial regulations, earlier diagnosis especially in men and a reduction in smoking. However, this is variable with increased incidence and mortality rates in Poland and Denmark[12]. (Figure 1.2)

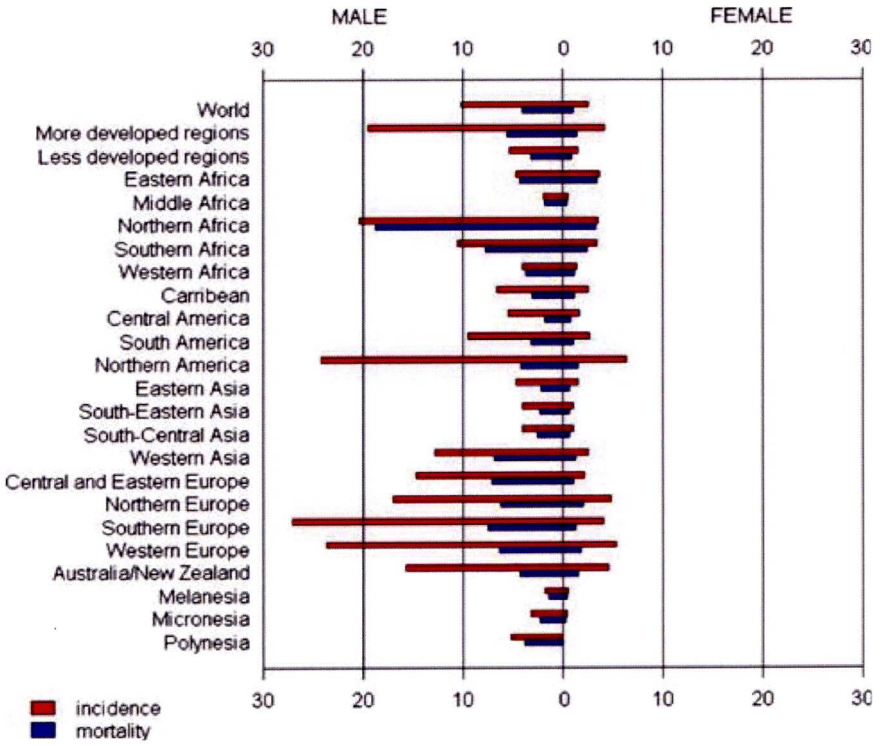


Figure 1.1: World--Age standardized incidence and mortality rates for urinary bladder cancer per 100,000[13]

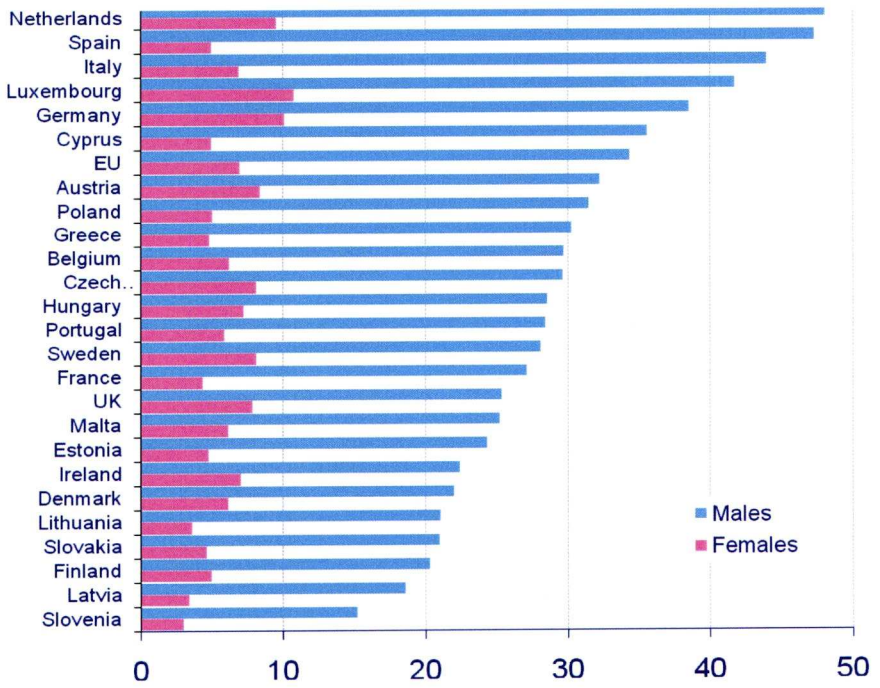


Figure 1.2: Age-standardised (European) incidence rates by sex-2002 estimates[14]

In the British Isles, urinary bladder cancer remains the fourth commonest cancer in men and the tenth most common cancer in women with more than 10 000 new cases reported in 2005 and almost 5000 deaths from urinary bladder cancer in 2007[14]. Mortality rates are markedly higher in the older age groups with 90% of both male and female deaths occurring after 65years of age. Cancer research UK[14] have analysed mortality statistics over a 35year period. Male mortality is reported to have fallen by 30% from 12.2 per 100 000 population in 1992 to 8.1 per 100 000 population in 2007 (Figure 1.3).

A 60% decrease in mortality from 12.8 to 5.0 per 100 000 population was noticed in men aged between 45 and 64 years. In females, the reduction in mortality rates are less marked with rates reducing from 3.5 per 100 000 in the 1970s/early 1980s to 2.8 per 100 000 in 2007 (Figure 1.4).

Again, the largest reduction has been seen in the 45-64 age group with rates falling from 3.8 per 100 000 in 70s/80s to 2.0 per 100 000 in 2001. One of the confounding factors in epidemiological studies is the differences in registration and reporting of low grade tumours. This makes accurate comparisons between countries imprecise.

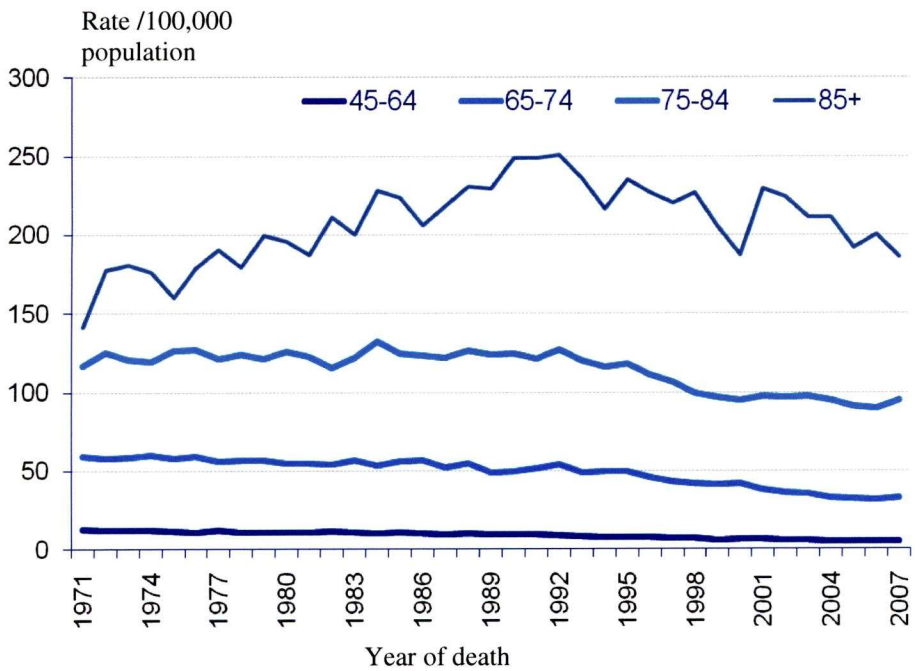


Figure 1.3: Age-specific mortality rates, males, UK 1971-2007

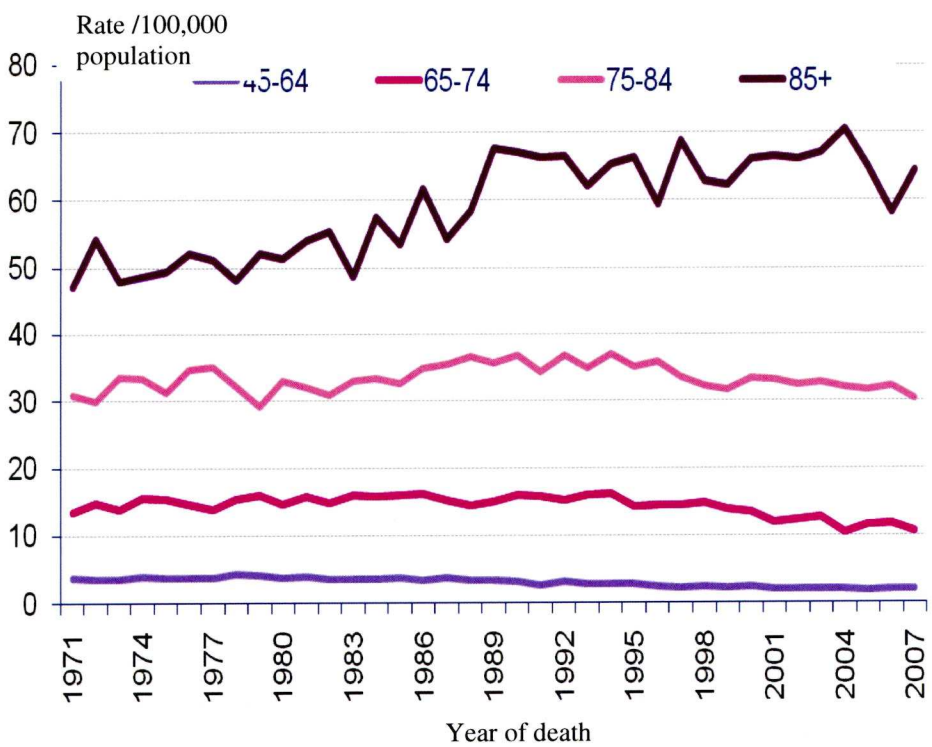


Figure 1.4: Age-specific mortality rates, females, UK 1971-2007

1.2: WHO/ ISUP Consensus classification:

The difficulties in accurate histological reporting were recognised by the World Health Organisation (WHO) and the International Society of Urological Pathologists (ISUP). In 1998, these two groups jointly published the WHO/ISUP consensus classification of urothelial (transitional cell) urinary bladder neoplasms. The benefit of this modified system was a universally acceptable classification which also took into consideration the nomenclature of pre-neoplastic lesions. In 2004, this classification system was adopted in Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs, one of a series of WHO Blue Books for the classification of tumors[15]. This version is known as the 2004 WHO classification and is essentially the same as the 1998 WHO/ISUP system (Table 1.1).

The WHO 1973 and WHO/ISUP 1998 classifications are two duplicate systems that remain the bench mark reporting tools used in histopathology to characterise urothelial lesions. These systems are not a direct correlation except at the extremes of classification where this one-to-one correlation holds true.

- Normal
    - Normal*
- Hyperplasia
    - Flat hyperplasia
    - Papillary hyperplasia
- Flat lesions with atypia
    - Reactive (inflammatory) atypia
    - Dysplasia
    - Carcinoma in situ#
- Papillary neoplasms
    - Papilloma
    - PUNLMP (papillary urothelial neoplasms of low malignant potential)
    - Papillary carcinoma, low grade
    - Papillary carcinoma, high grade

*May include cases formerly diagnosed as mild dysplasia

#May include cases formerly diagnosed as severe dysplasia

Table 1.1: WHO/ISUP consensus classification (2004) of urothelial lesions[16]

1.2.1: Normal urothelium:

The urothelium is routinely exposed to a wide range of toxins that are excreted as a result of the normal physiologic functions of the kidney and the storage role of the urinary bladder. A slight degree of architectural turbulence could be considered normal (Figure 1.5). Over the years, there has been a tendency to over-diagnose 'dysplasia'. The consensus group decided to drop the term "mild dysplasia" deciding that these flat lesions with minimal cytological atypia and architectural disarray should be described as normal. The reason behind this clarification is that these lesions without cytological atypia lack premalignant potential.

1.2.2: Hyperplasia

1.2.2.1: Flat urothelial hyperplasia:

This is defined as markedly thickened mucosa but without cellular atypia (Figure 1.6). The previous suggestion for the urothelium to exceed seven cell layers by thickness[17] has been amended to requiring obvious urothelial thickening. There is no data to suggest that this lesion is pre-neoplastic but can be seen in adjacent mucosa to low-grade lesions.

1.2.2.2: Papillary urothelial hyperplasia:

These are asymptomatic lesions usually found during cystoscopic surveillance for urothelial carcinoma[18]. It is characterised by variable thickness urothelium with fluctuating growth (Figures 1.7, 1.8).

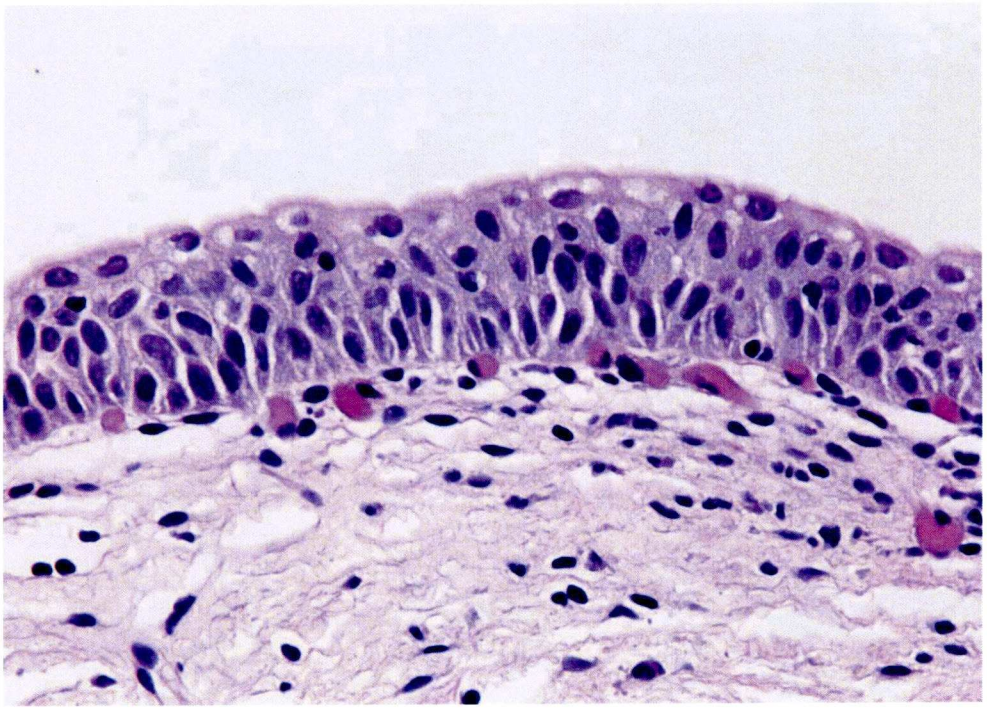


Figure 1.5: Normal urothelium. Slight nuclear irregularity still falls within the spectrum of normal urothelium.

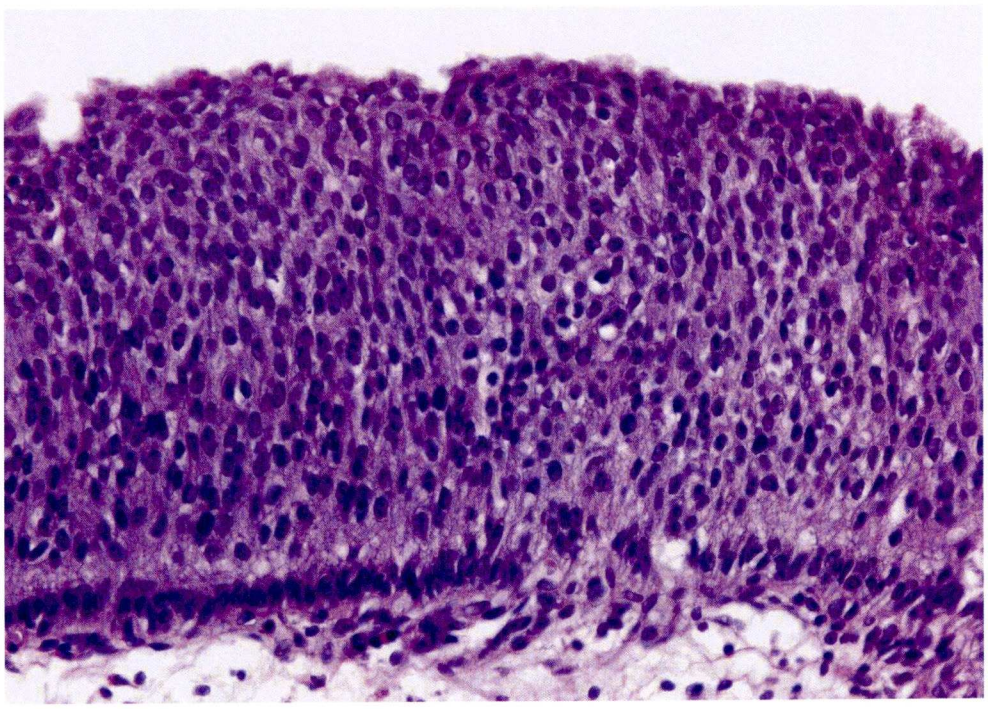


Figure 1.6: Flat Urothelial Hyperplasia

Figure 1.7: Papillary Urothelial Hyperplasia without atypia

Figure 1.8: Papillary urothelial hyperplasia without atypia. Nuclear architecture and cytological arrangement appear normal

These lesions distinctly lack fibrovascular cores. This diagnosis *per se* does not place a patient at risk of developing carcinoma but cystoscopic follow up is advised. However, if this is diagnosed in patients with a history of papillary neoplasms, it is associated with an increased risk of recurrence of papillary carcinoma[18].

1.2.3: Flat lesions with Atypia:

Patients with reactive atypia have a history of intravesical therapy, calculi or instrumentation with consequent inflammation. The WHO/ISUP (1998) clarified the histological picture for reactive (inflammatory) atypia from dysplasia. Reactive (inflammatory) atypia consists of nuclear abnormalities occurring in inflamed urothelium either acute or chronic. This is characterised by a mild increase in nuclear size and central prominent nucleoli within a vesicular nucleus (Figure 1.9). There is absence of nuclear hyperchromasia, pleomorphism and chromatin irregularity. Mitotic figures maybe frequently present.

1.2.4: Dysplasia

Dysplasia or Low-grade intraurothelial neoplasia refers to urothelial lesions with noticeable significant architectural disarray and abnormal cellular structure[19] (Figure 1.10).

Studies have shown that dysplasia could be a precursor of invasive carcinoma[20] and are typically seen in bladders with urothelial neoplasia and uncommon in bladders without it[21-23].

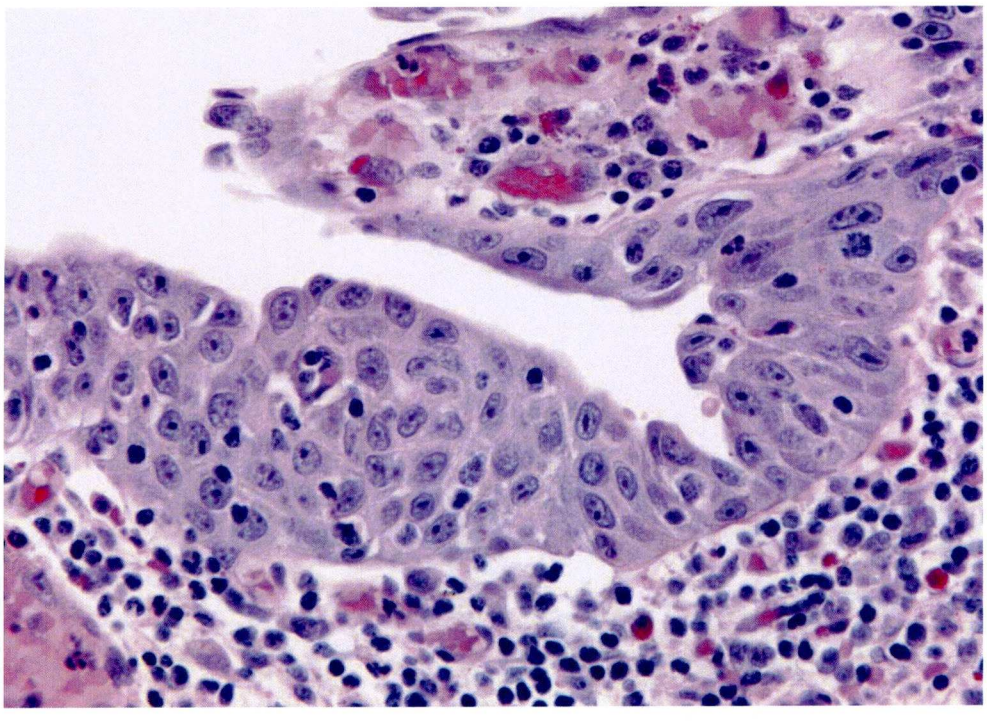


Figure 1.9: Reactive Urothelial atypia

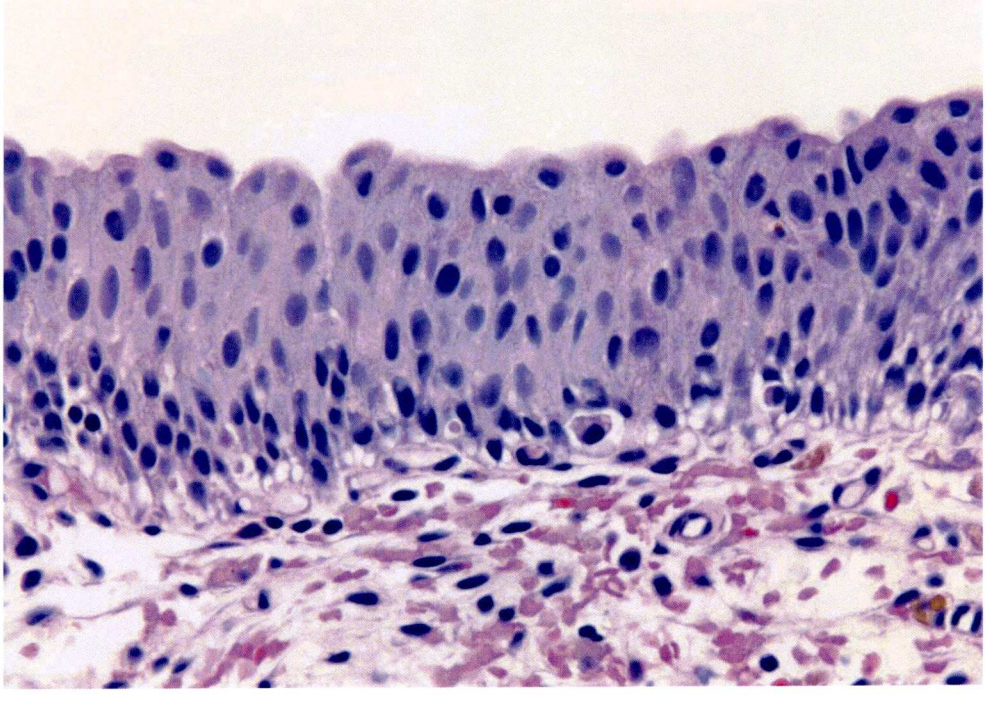


Figure 1.10: Urothelial Dysplasia

Management of dysplasia is varied among differing urologists. It is usual practice to treat dysplasia with intravesical chemotherapy only if it occurs in association with and/ or a history of urothelial neoplasia or carcinoma insitu as these patients are at a higher risk of recurrence and progression[20 24 25]. However, urothelial dysplasia is a precursor lesion to invasive carcinoma in some patients. This is supported by findings of concurrent dysplasia and carcinoma, absence of dysplasia in non-neoplastic bladders and the genetic similarities with carcinoma in-situ[19 21-23].

1.2.5: Carcinoma in situ (CIS):

The WHO/ISUP system has attempted to clarify the histological confusion on carcinoma in situ. Carcinoma in situ or high-grade intraurothelial neoplasia is a flat urothelial lesion which in the past has been labelled as marked atypia or severe dysplasia. It is considered a high-grade lesion and a defined precursor of invasive carcinoma; characterised by cells with large, irregular, hyperchromatic nuclei and nuclear membrane irregularities (Figure 1.11). This maybe present through the entire epithelial thickness. The Consensus committee[16] have suggested that carcinoma in situ is often under diagnosed and offer suggestions why:

1. Full thickness cytologic atypia is not required for a diagnosis of carcinoma in situ. Patterns of carcinoma in situ include those with scattered carcinoma in situ cells, Pagetoid spread of carcinoma in situ, and cases where the fragile epithelium may be disrupted either

spontaneously or by the biopsy so that only a few residual cancer cells remain on the surface (clinging carcinoma in situ)[26]

2. Carcinoma in situ cells do not always have a high nuclear/cytoplasmic ratio
3. An umbrella cell layer may still be present in carcinoma in situ
4. There is a spectrum of cytologic atypia within carcinoma in situ. However sub-classifying carcinoma in situ based on the cellular ploemorphism should be avoided as this should be compared in conjunction with surrounding normal urothelium

In making the diagnosis of carcinoma in situ, the most important parameters are the nuclear features.

1.2.6: Papillary Urothelial Neoplasms:

Prior to the Consensus criteria being developed in 1998 and consequently confirmed in 2004, there were varied yardsticks in defining papilloma and low grade papillary carcinoma as well as in grading papillary urothelial carcinoma. The architectural criteria describing the various grades in the sub classification is one of the important benefits from the WHO/ISUP system. These criteria are based on the papillae structure and cellular features. The cellular features include nuclear size and shape, chromatin content, nucleoli, mitoses and umbrella cells. The classification system brought in advantageous similarity with cytology.

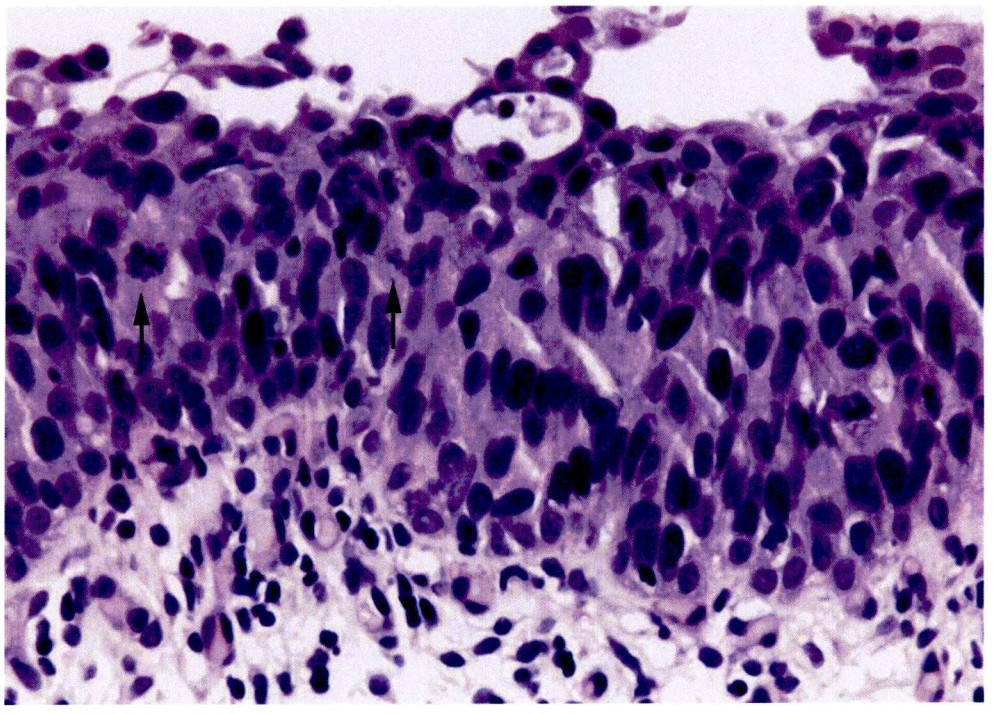


Figure 1.11: Carcinoma in situ with hyperchromatic nuclei and numerous mitotic figures (arrows)

Figure 1.12: Benign Urothelial Papilloma

1.2.7: Papilloma:

Strict histological criteria have been defined for the diagnosis of papilloma. The defined appearance would be a discrete papillary growth with a central fibrovascular core and lined by normal looking urothelium (Figure 1.12). This is a rare benign condition exemplified as small isolated growths primarily in younger patients. Inverted papilloma is a variant of papilloma but with some similar features of the exophytic type. Resection is curative in both with very low recurrence rates[27].

1.2.8: Papillary Urothelial Neoplasms of Low malignant potential (PUNLMP):

These are papillary urothelial lesions that do not have cellular features of malignancy but have thickened urothelium and/or nuclear enlargement (Figures 1.13, 1.14). There is no nuclear dysmorphism unlike low grade papillary tumours. In the PUNLMP lesions, the distinct difference from papillomas is a thicker urothelium and with enlarged nuclei. Mitotic figures if present are limited to the basal layer. The importance of this group labelled as neither benign nor overtly malignant stems from the increased risk of developing recurrent or new papillary lesions which are usually of the same histology but lower rates than low-grade papillary cancers[28-30]. However, occasionally the subsequent lesions are of higher grade and could progress[31]. This category gives the clinician the opportunity not to label a patient as with cancer but ensures that a closer follow-up regime is instituted.

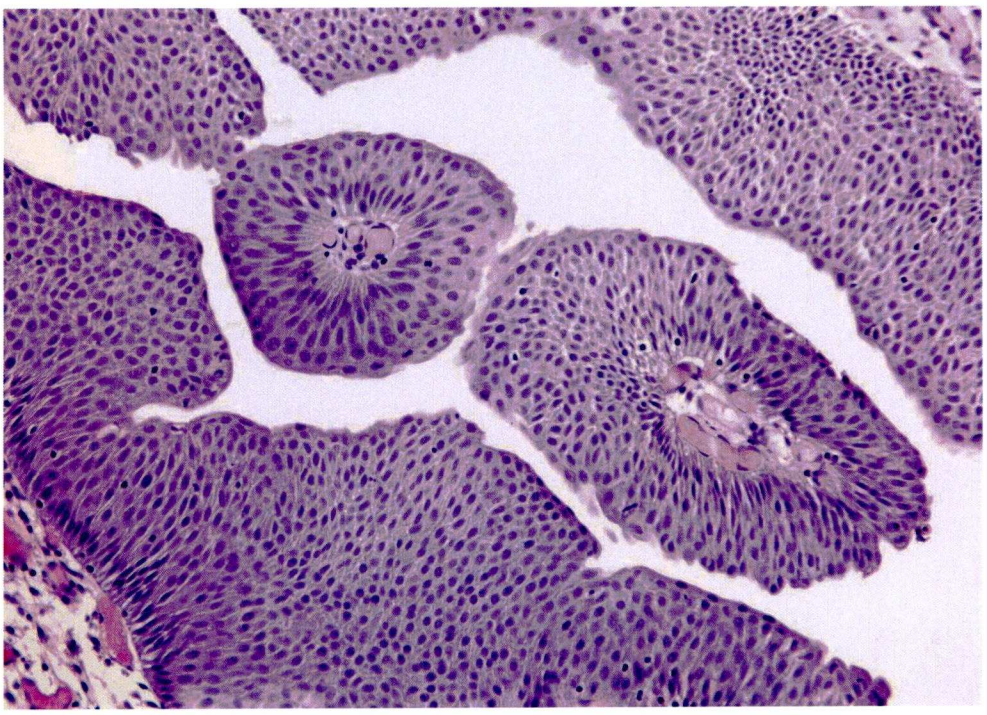


Figure 1.13: Plain view of papillary urothelial neoplasm of low malignant potential

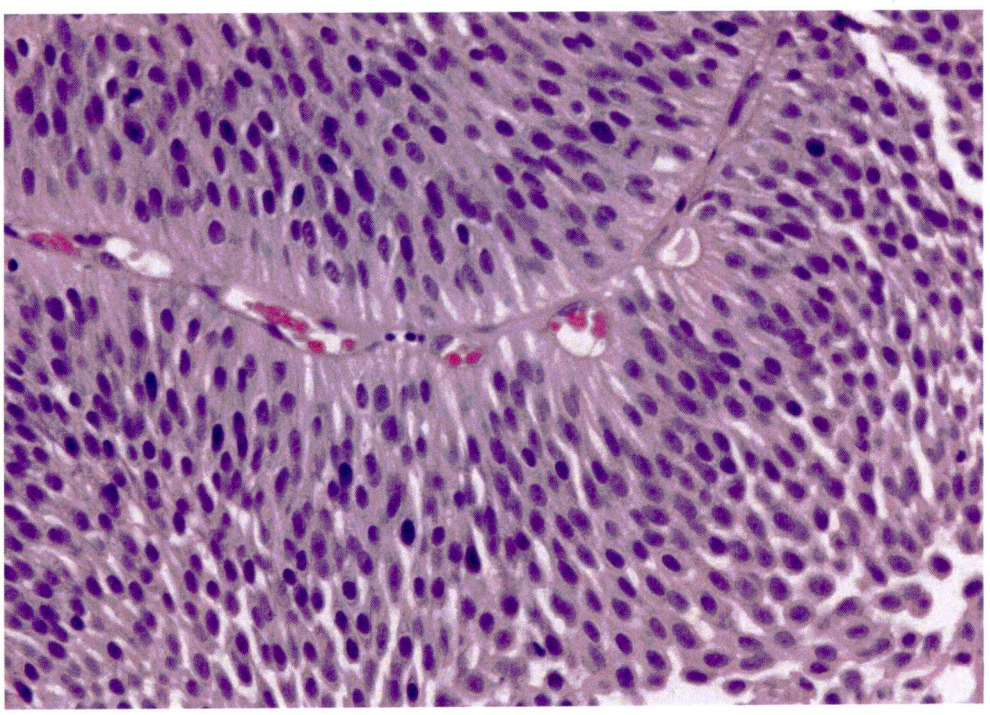


Figure 1.14: Cytologic appearances of papillary urothelial neoplasm of low malignant potential

1.2.9: Low-grade & High-grade papillary urothelial carcinoma:

One of the significant pitfalls of the WHO 1973 classification system was the intermediate group (WHO grade 2) which was often the default diagnosis for pathologists In an attempt to simplify this issue, the WHO/ISUP system classifies papillary urothelial carcinoma into only two grades[31]

1.2.9.1: Papillary urothelial carcinoma, Low grade:

Low-grade papillary urothelial carcinoma has an orderly appearance with minimal architectural variability and/or cytological changes which are easily discernable at low magnification (Figure 1.15). The significant findings include variations in nuclear size, shape, polarity and chromatin texture. Mitotic figures are infrequent and usually seen in the lower half of the urothelium. The difficulty in differentiating papillary neoplasms of low malignant potential from papillary carcinoma of low grade is significant as the histological differences maybe subtle. However, some cells within papillary low grade carcinoma are overtly neoplastic, whereas cells in tumours of low malignant potential lesions show in general only nuclear enlargement. Low grade papillary carcinomas may invade the lamina propria, and have a low (<5%) risk of further progression but can frequently recur[16].

In the presence of low grade urothelial carcinoma, it is important to understand that a variable spectrum of the histological patterns could be present concurrently. The tumour should be graded based on the highest

grade present. However, the current practice is to disregard minute areas of highest-grade tumour. The significance of this tendency on disease prognosis has yet to be elucidated.

1.2.9.2: Papillary urothelial carcinoma, High grade:

High-grade papillary urothelial carcinoma is an aggressive tumour with marked architectural and cellular asymmetry. The cells appear irregularly clustered with disorganised epithelium and a spectrum of cytological pleomorphism. The nuclear chromatin tends to be clumped and nucleoli may be prominent. Mitotic figures, including atypical forms, are frequently seen at all levels of the urothelium (Figure 1.16). The consensus report suggested that instead of sub-classifying based on the variable level of cytologic atypia, it would be an option for pathologists to comment on the degree of nuclear anaplasia. Moderate to marked nuclear pleomorphism, abnormal mitotic figures, clumped chromatin and nucleolar prominence all favour high-grade over low-grade carcinoma.

There is a higher risk of progression with high grade papillary urothelial carcinomas varying from 15% to 40%[16]. These tumours also have a high risk of association with invasive disease at the time of presentation[28-30]. In equivalence to the high grade cytologic atypia within these lesions, the surrounding flat urothelial mucosa may also demonstrate CIS.

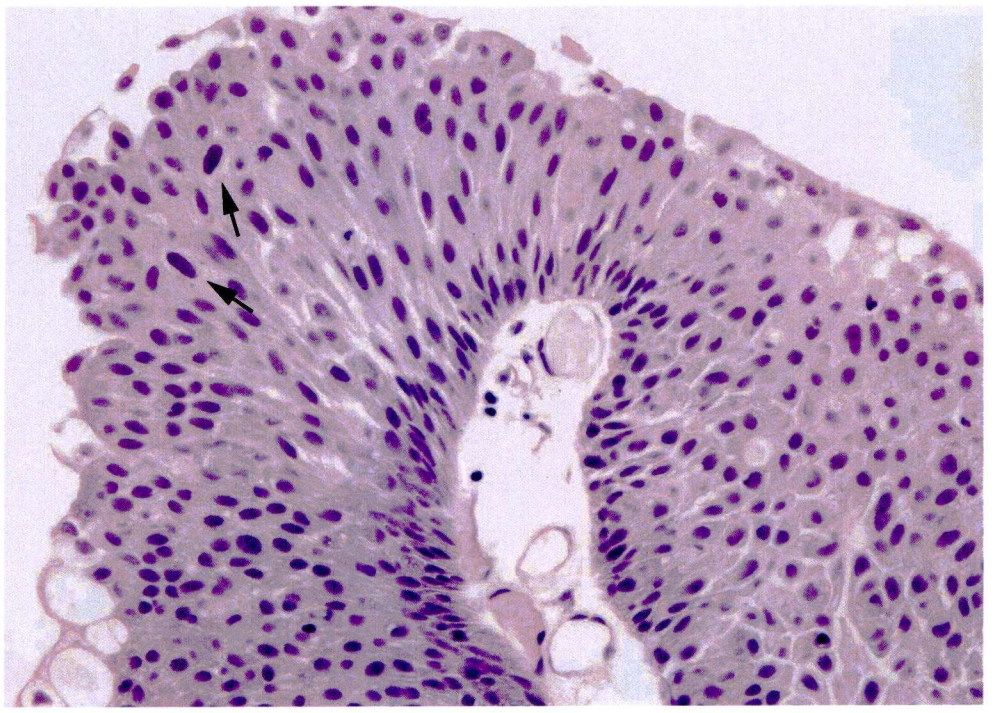


Figure 1.15: Low grade papillary urothelial carcinoma. Low magnification-slight but definite nuclear pleomorphism with scattered hyperchromatic and enlarged nuclei (arrows) relative to surrounding nuclei

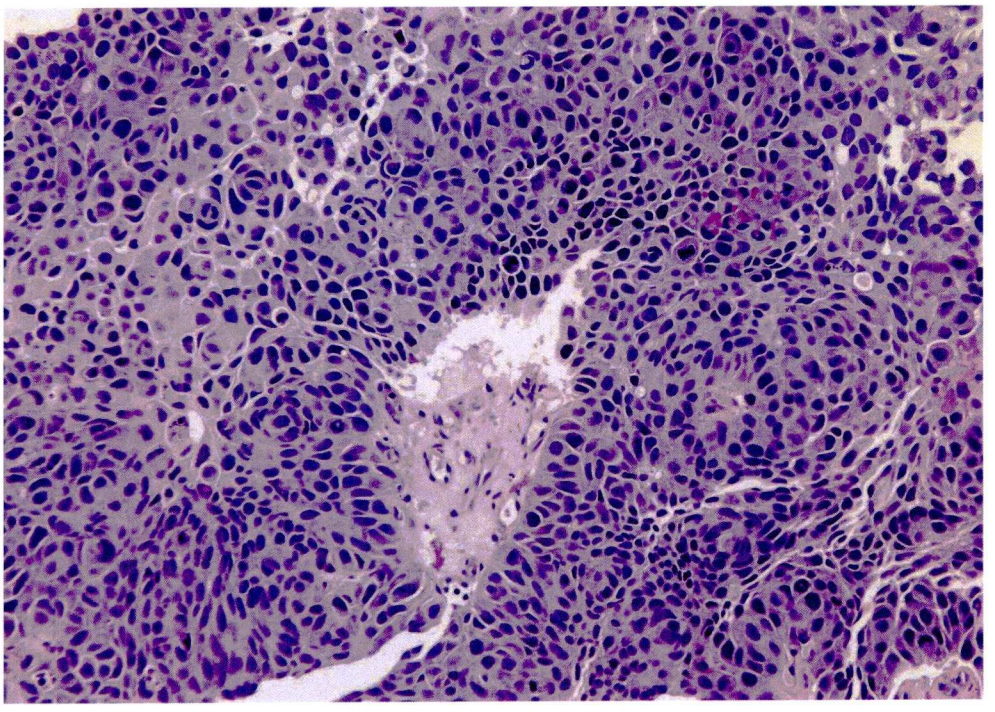


Figure 1.16:High-grade papillary urothelial carcinoma with marked architectural disorder

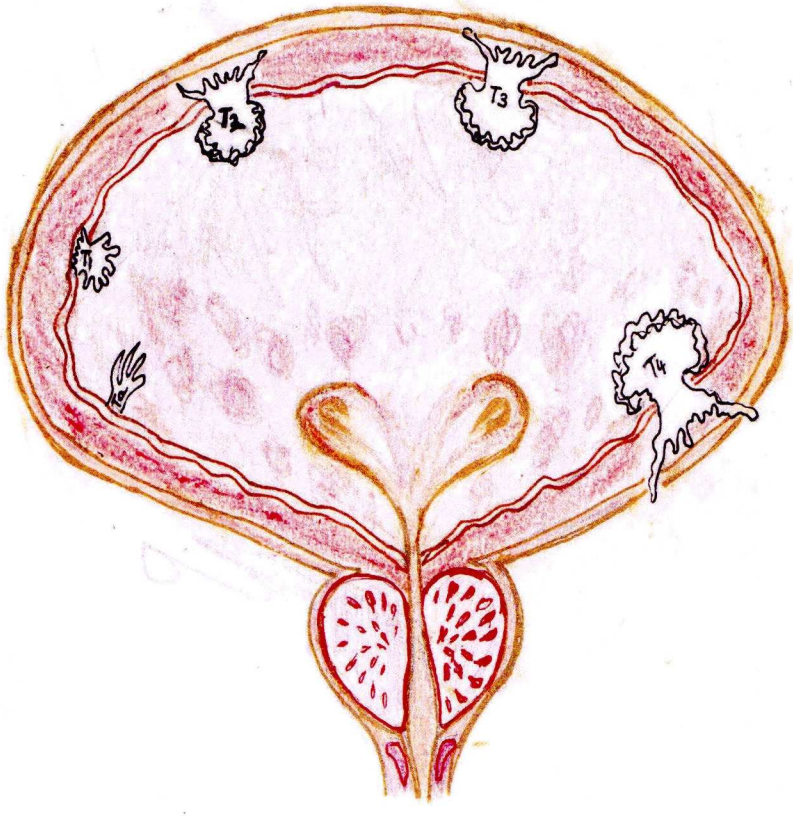
1.3: Invasive Urothelial Neoplasia:

The descriptive nomenclature regarding invasive disease has also been confusing. The importance of quantifying depth of invasion in planning treatment and prognostic significance resulted in the WHO/ISUP committee developing several recommendations to provide clinicians with clear-cut essential information.

1.3.1: pT_1 tumours:

The 2002 TNM (Tumour, Lymph nodes and Metastases) staging system (Figure 1.17) defines pT_1 tumours as those invading the lamina propriae which is the connective tissue layer underneath the mucosa. There may also be an interrupted layer of smooth muscle (muscularis mucosae)[32]. Invasion of the lamina propria is characterised by urothelial cellular nests which have breached the epithelial basement membrane. Another feature of invasive tumour that is not always present is an associated desmoplastic or inflammatory stromal response.

There is a significant observer variability in diagnosing pT_1 disease with concurrence varying from 61%-88% [33 34]. Kirkali *et al.* [6] reported that experienced genitourinary pathologists down-staged 56% of the tumours to pTa on review, and up-staged 13% to muscle invasive disease. Down staged tumours showed less degree of progression than pT_1 tumours (17% vs 25%) with consequent prognostic implications[6].



Key: Ta-Non-invasive confined to urothelium; T_1-invades lamina propria;
T_2-invades bladder muscle; T_3-invades extravesical fat;
T_4-invades pelvic/abdominal wall/neighbour organs

Figure 1.17: Correlation of the extent of tumor invasion with the TNM staging system of carcinoma of urinary bladder

With extension of invasion, the cancer cells may exhibit eosinophilic cytoplasm at the advancing edge of the infiltrating clusters and eventually invades the smooth muscle bundles of the detrusor. The important discriminator remains the differentiation between invasion of the muscularis mucosae and the muscularis propria as this has treatment planning implications.

1.3.2: pT_2 tumours:

The urinary bladder muscle (detrusor) wall contains larger muscle bundles referred to as the muscularis propria. Muscularis propria invasion (pT_2) is diagnosed when tumour is seen infiltrating thick smooth muscle bundles. The depth of invasion can be difficult to appreciate especially in resection specimen. This includes cases where there is extensive tumour with scattered slips of muscle could give the appearance of muscularis mucosae or could be markedly distorted and disrupted muscularis propriae. Also, there are occasions when adequacy of muscle in the specimen is contentious requiring a restaging resection.

The TNM staging sub-classifies pT_2 into pT_2a; cancer invading <50% of the muscularis propriae depth and pT_2b where cancer invades more than ½ of the muscularis mucosae. The clinical benefit of this sub classification has been questioned as studies have shown no survival difference[35-37]. Roehrborn *et al.* [36] (65% for pT_2a, 61% for pT_2b) and Pollack *et al.* [37] (78% for pT_2a, 77% for pT_2b) reported similar 5-year survival in the two groups.

Cheng *et al.*[35] in a multivariate analysis reported that tumour size rather than depth was predictive of distant metastasis-free and cancer-specific survival in patients with muscularis propriae involved cancer.

1.3.3: Extravesical disease:

pT_3 & pT_4 tumours refer to extravesical (locally advanced) disease with pT_3 denoting peri-vesical disease (a-microscopic, b-macroscopic) and pT_4 denoting tumour that has invaded other organs such as the prostate, uterus, vagina, pelvic wall or abdominal wall.

The WHO/ISUP consensus classification is a nomenclature system for urothelial lesions that can be understood by multi-disciplinary clinicians. It has been an attempt to produce clarity in the staging so as to improve the prognostic predictability as well as the treatment plan. Over a period of time, this system is likely to be refined and revised.

1.4: Risk Factors:

There has been an improvement in survival rates in patients with urothelial carcinoma. Despite this, the socioeconomic burden remains high with the total cost of bladder cancer estimated at £55 million between 2001 and 2002; almost 60% of which was associated with non-muscle invasive urothelial cancer[38]. Sangar *et al.*[38] postulated the reasons for this high cost of non-muscle invasive urothelial cancer (£8350 per patient) on incidence, long survival requiring life-long follow-up and the frequent recurrences requiring repeated resections and treatment. Grasso has suggested implementing various strategies to reduce the burden of bladder cancer. Prevention whereby occupational risk factors need to be understood and protocols developed to modify/ reduce exposure risk. Early and accurate diagnosis with the help of advanced techniques to stage bladder cancer more precisely enables early intervention in patients before non-muscle invasive urothelial cancer progresses to an aggressive and life-threatening disease.

1.4.1: Smoking

Cigarette smoking is the most important modifiable risk factor for urinary bladder carcinoma. The extent to which smoking can be held accountable of developing bladder cancer is variable. There has been a worldwide paradigm shift in bladder cancer incidence with a decrease in incidence and mortality in western communities over the last few decades associated with

a decline in smoking prevalence in both men and women. There is also a shift in the tobacco epidemic towards the developing world with the WHO reporting an increase of up to 16% in these countries[39]. The most important risk factor in present day population based studies is tobacco smoking. Gandini *et al.*[40] in their meta-analysis estimated an on overall relative risk of urothelial cancer between current and former smokers of 2.77 and 1.72, respectively. In Europe, an estimated $^{2}/_{3}^{rd}$ of cases in men and $^{1}/_{3}^{rd}$ of the cases in women are attributable to smoking as a contributory cause[41]. Brennan *et al.*[42] in a pooled European wide study suggest that there is an increase in bladder cancer risk linearly with duration of smoking. This crests at an average of 15-20 cigarettes a day. There are several suggested explanations for this threshold effect with number of cigarettes and risk for bladder cancer. Reports suggest that high volume smokers (≥20 cigarettes/day) inhale less with individual cigarettes[43]. The metabolic polymorphisms also involved may exert more prominent effect at low levels of exposure[44]. However, these reports are in contrast to the more striking association of smoking with lung cancer where epidemiological studies have failed to show an association with number of cigarettes smoked but with a greater than linear increase with smoking duration[45] which suggest that the group of carcinogens maybe different in these two disease groups. Tobacco consumption may be involved in an early irreversible stage in the carcinogenic process based on the observation of a higher risk among long-term smokers (≥25years); also the tobacco consumption amount rather than number of cigarettes could be the critical factor[42]. This is corroborated by studies which report very high adjusted odds of greater than five in black

tobacco smokers[46]. Black tobacco is suspected to be twice as carcinogenic as blond tobacco[47 48] with experimental data finding more concentrated aromatic amines and nitrosamines[49] as well as urinary mutagenicity and 4-aminobiphenyl hemoglobin adducts levels[50] higher in black tobacco smokers. This could be the explanation for higher odds ratio for developing bladder cancer in countries such as Italy and Spain[47] in comparison with the U.K. and U.S.A.

1.4.1.1: Marijuana:

Marijuana use has seen a rise in the 80s and 90s with a stable rate of 4% over the last decade[51]. There has been a significant increase in dependence and marijuana-related disorders in the last few years[52]. This has been attributed to the delta-9-tetrahydrocannabinol (THC) in marijuana[53] which has tumour promoting properties[54]. THC levels in the urine have a half-life of almost three days compared to 12 hours for nicotine metabolites making it a more potent stimulant of urothelial malignant transformation[55 56]. Marijuana also induces cytochrome P450 enzyme pathways which potentiates the deleterious effects of nitrosamines and aromatic hydrocarbons from tobacco smoking[57 58].

1.4.1.2: Passive smoke exposure:

This is a difficult entity to quantify. Zeegers *et al.*[59] in a large prospective cohort study, found no significant association between secondhand smoking

and an increased bladder cancer risk. However, there are other studies which have suggested a significant involvement. Alberg *et al.*[60] in a prospective cohort study with over 40 years follow up showed a 2.3 times increase in bladder cancer risk in women exposed to secondhand smoke in comparison to those not exposed with some reporting a dose response effect for the amount of secondhand smoke exposed[61]. Childhood exposure to smoke also increased the risk of developing bladder cancer as an adult[62]. Others feel that the exposures in utero and through mother's milk which have higher levels of nicotine in smoker mothers maybe responsible[63].

1.4.1.3: Cessation of smoking:

The risk of developing bladder cancer in former smokers decreases dramatically with reduction of 40% within 1-4 years after cessation[42] with the risk reduction improving with the number of years following cessation[59].

1.4.2: Occupational Risk:

Almost a fifth of bladder cancers can be directly attributed to be occupational exposure[64]. Occupational risk has been observed in the occurrence of bladder cancer since the early 80s when the increased incidence of bladder Ca was noted in the Yorkshire belt. Boyko[65] analysed data from a large case control study on chemical dye workers in West Yorkshire reporting 2-3 times increased risk in those exposed to aryl-amines. Dose-response relationship for calendar years of exposure demonstrated a significantly increased risk in those exposed for over 30 years. β-naphthylamine and benzidine were banned in the United Kingdom about 1950 and 1962, respectively. This along with 4- aminobiphenyl are the main agents that have been associated with bladder cancer with high relative risks[66]. In the UK, revised working practices in place after 1960 have helped reduce the risk.

One of the major studies in assessing the occupational risk for developing bladder cancer was published in the Journal of the National Cancer Institute in 1970 by Anthony *et al.*[67]. They reported on the whole life occupational history of more than a thousand patients with bladder cancer in the Leeds area. This was a case controlled study matched for age, sex, habitat and smoking habits. Results confirmed the risk to dye workers and revealed risks to medical workers (mainly nurses), to tailors, tailors' pressers, and some groups of engineers and textile workers (associated with long-term employment only), and possibly also to hairdressers and tailors' cutters.

Tumour occurred at younger ages in men who had been employed as dye workers, tailors' cutters (P = < 0.025), or hairdressers (not significant)[67]. This has been confirmed by other studies with reports of an increased odds ratio for developing urothelial cancer in people dying cloth or dye manufacturing[68 69], tailors[68] with a positive trend with increasing duration of employment[70].

Studies including a meta-analysis[71] have shown an increased risk in painters[68] with benzidine based azodyes, one of the important carcinogenic risk factors[72] which may release aromatic amines in humans[73], leather workers, shoe makers and in the rubber tyre industry[71 74].

A Canadian study[68] on risk of bladder cancer in men employed for more than six months in the chemical industry have reported increased risk in those exposed to diesel or traffic fumes and drill press workers. Polycyclic aromatic hydrocarbons (PAH), a product of petroleum combustion could be responsible for a higher incidence reported in those working in the petrochemical industry, blacksmiths & mechanics[75]; those exposed to oil, gasoline, chemical materials, kerosene and asphalt.
However, despite paint and ink being linked solvents, no studies have shown a significant association with urinary bladder carcinoma although the difficulty might lie in the diverse nature of these occupations with variable exposure [76 77].

Aluminium smelting (Soderberg process)[78] , coal mining or exposure to tar, pitch or asphalt are other settings where significantly raised risks of developing urothelial cancer have been reported with exposure to PAH[78]. PAH consist of fused aromatic rings, and being lipophilic are metabolised in the liver by the cytochrome P450 system[79]. Various PAHs; if not rendered labile can form PAH-DNA adducts which can lead to mutations in DNA during cell division, resulting in mutations and consequent carcinogenesis if this occurs in critical genes[80].

Another occupational group that has been studied are the agricultural and horticultural workers. This was pertinent due to their increased exposure to pesticides and weedicides. This group included workers involved in pesticide manufacture and crop sprayers. Studies have suggested that there may be an increased risk[81] but this has not been substantiated[76].

Health workers especially nurses have been reported to have an increased risk. Most studies[77 82] have failed to show an associated risk in either sex, except a case series[83] were non-smoking male physicians were found to have a nine- fold relative risk.

Hairdressers, barbers, tailors and weavers have received extensive attention especially in light of the evidence concerning synthetic dyes. Only Kabat *et al.* (cohort study) have reported an increased risk for urothelial cancer[84]. However confounding factors such as smoking were not taken into account. Exposure to synthetic materials for >10 years and launderers have been

reported to have a higher risk of urothelial cancer of up to two times or more. This is explained by exposure to dyes and synthetic materials. Domestic helpers and Cleaners are also in this category.

In summary, employment studies convey an increased risk for urothelial cancer in professions involved in manufacturing or using dyes or related solvents. PAH has been reported to be the main culprit in the increased noticed risk in people involved directly or indirectly with the petrochemical industry. Most other occupations do not appear to be associated with an increased risk towards developing bladder cancer.

1.4.3: Food and Beverages:

The effect of dietary parameters in cancer risk has been long debated. Very few studies have reviewed the role of diet in urothelial cancer. A case-control study from Uruguay[85] assessed a variety of food sources reporting significantly elevated risks with salted and barbecued meat as well as fatty foods. Salted meat contained high levels of sodium chloride which has previously been reported to be a carcinogen[86], barbecued meat containing heterocyclic amines; another powerful carcinogen[87]. Frequent consumption of canned foods attributable to the loss of vitamins in the canning process[88] and fried foods[86] were also implicated.

Anti-tumour effects have been reported with the ingestion of various food items. There was a significant protective effect with cooked vegetables, citrus fruits, potatoes and all tubers[85]. Cruciferous vegetables (cauliflower and cabbage) have been found to have a high isothiocyanate levels which have a chemo-preventive role in bladder cancer[89]. Green vegetables and carrots seem to offer protection with Wakai *et al.*[90] suggesting that a high intake of green-yellow vegetables could decrease the bladder cancer incidence by almost 40%.

Beverages intake has been difficult to quantify. Claude *et al.*[88] in a matched case-control study of over 450 patients report their results on a range of fluids. A significant odds ratio for drinking more than four cups of coffee daily of 1.3 was found. However, this association was not seen in non-smokers. Beer consumption of more than one imperial pint a day increased relative risk two fold after controlling for smoking[88 91]. However others have not found any association with coffee or high-proof spirits[92-94]. This could be explained by the varying control groups and the different spirits consumed. Patients with excessive alcohol intake might also have nutritional deficiencies which maybe contributory. This corroborates the findings from the Danish micronutrient intake study[95] from which dietary beta-carotene (precursor of Vitamin A) have been shown to be associated with a 18% lower risk per 500microgram higher intake. This vitamin A precursor potentiates dietary anti-oxidative factors which may be the true effect modifiers. A large prospective Swedish study on high intake of cultured milk suggest that ≥two servings of cultured milk was associated

with a 38% lower risk[96]. Others have reported decreased risk with consumption of fermented milk products and yoghurt[97 98]. Lactic acid bacteria is considered the modulating factor by an unclear mechanism on the immune system[99] and also suppressing food-derived urinary mutagenicity in humans[100]. A Japanese cohort study of people who consumed milk on a daily basis had a significant (53%) lower mortality due to bladder cancer[101] with inconsistent evidence from other cohort studies[102-104].

Urothelial carcinoma is a multifactorial disease with both environmental (and occupational) dietary and genetic factors involved in the development and progression of this disease. However, the reported studies of carcinogenic activity of various environmental carcinogens have provided insubstantial or weak evidence precluding any strong suggestion that risk modification would enable a reduction in incidence. This has resulted in researchers reviewing the genetic epidemiology of urothelial cancer. Genetic variants in nucleotide excision and double strand break DNA repair pathways and modern tissue microarray studies support the concept of molecular alterations affecting "genetic pathways" leading to carcinoma. Phenotypic and genotypic profiling of urothelial tumours have become a powerful approach that provides valuable information on the biological status of individual bladder cancers which is detailed in the next chapter.

Chapter 2:

Phenotypic and genotypic pattern in Human Urinary Bladder Carcinoma

In the United Kingdom, transitional cell carcinoma (urothelial carcinoma) is the most common type of urinary bladder carcinoma accounting for 90% of the 10,091 bladder cancer cases diagnosed in 2007[105]. These cancers become clinically apparent as either papillary lesions, which are less likely to invade rapidly or deeply into the bladder wall but have a high tendency to recur necessitating multiple tumour resections; or the less common flat lesions which directly invade the bladder wall and are related to poor prognosis[106]. Presently, the papillary tumours are classified into three morphological grades (UICC criteria) according to their architectural pattern, nuclear pleomorphism and mitotic figures. In contrast, morphologically flat lesions could be either reactive, dysplastic or neoplastic. Reactive lesions may contain inflammatory changes in the lamina propria beneath a hyperplastic or metaplastic urothelium. Cytological abnormalities occur in dysplasia and include cellular and nuclear pleomorphism restricted to the basal and intermediate layers. Urothelial carcinoma in situ (CIS) is frequently multifocal and characterised by a flat proliferation of disordered urothelial cells with marked atypia. Flat malignant lesions tend to be invasive and aggressive with a poor clinical outcome (Figure 2.1).

Following initiation of these different morphological types of urothelial neoplasms by changes within distinct spectra of genes, the genotypic and phenotypic factors which, thereafter, promote invasion and metastasis of individual bladder cancers are likely to be similar.

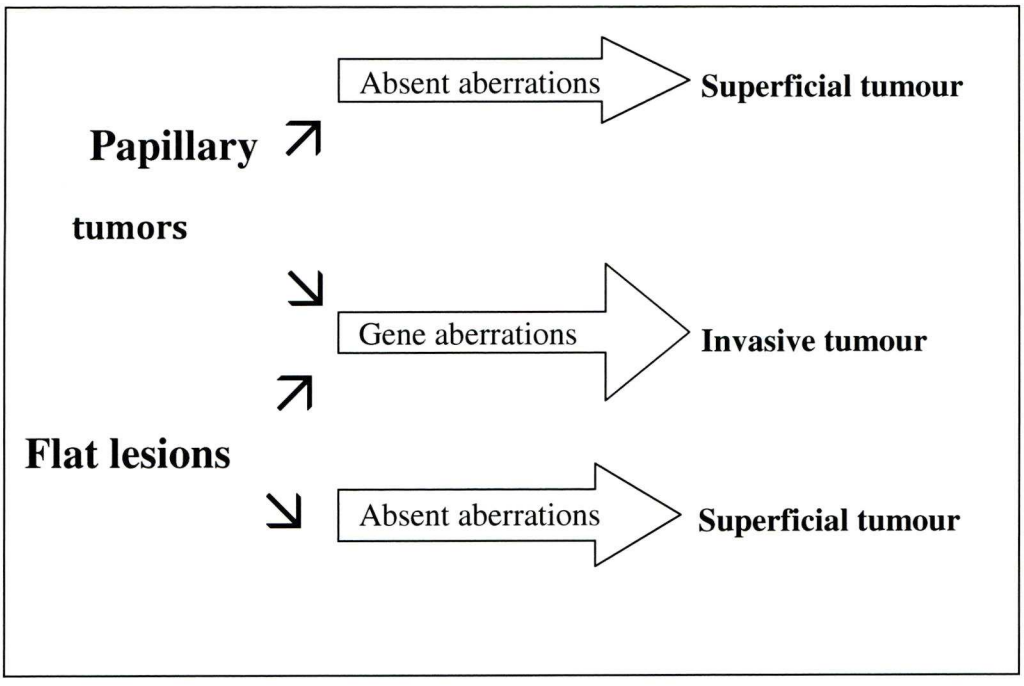


Figure 2.1 – Initial concept of the relationship between the origins of superficial and invasive tumours.

Invasive transitional cell carcinomas can arise de novo[107], occur following progression of a flat lesion[108] or develop from the progression of a superficial papillary tumour[20 109]. Nevertheless, identification of the invasive potential of particular papillary or flat lesions remains a dilemma, particularly in the pre-invasive stage. Morphologic similarities between superficially-invasive papillary tumours that are recurrence-prone and invasive tumours with deep bladder wall penetration presently confound diagnostic and prognostic prediction. Although genetically encoded, many of the factors that control the behavioural phenotype of an individual cancer are likely to be epigenetic.

Many studies have been conducted to identify genes associated with bladder cancer development[110]. Several molecular studies[111 112] of transitional cell bladder tumours have revealed characteristic chromosomal abnormalities. Spruck *et al.*[111] proposed two distinct molecular pathways for bladder cancer development with acquisition of particular chromosomal defects responsible for invasive capability. The genes considered permissive for malignant transformation comprised several oncogenes and tumour suppressor genes. Initially quiescent, proto-oncogenes may be activated by critical genetic events which can include mutation, altered expression due to hypermethylation or novel insertion of viral DNA. Such events could occur in isolation or following inactivation of a tumour suppressor gene, thus causing a disturbance in the cell-cycle leading to malignant transformation[110].

Two major chromosomes involved in bladder cancer, of both squamous and papillary types, are chromosomes 9 and 17[113 114]. Loss of heterozygosity (LOH) on chromosome 9, characterize 60-65% of transitional cell carcinomas and is considered by some to be the earliest event in the genesis of transitional cell bladder cancer[112 114]. Functional assays of transitional assays have demonstrated lack of protein expression of cells containing mutations of the p16 gene[115] or on the short arm (region p21) of chromosome 9[116]. A high frequency of allelic loss on chromosome 9p21 appears to be frequently involved in bladder cancer progression[117]. Damage to chromosome 17 is suggested to be a late event. LOH of chromosome 17 short arm was noted in 40% of bladder tumours, mainly of high grade and

high stage[118]. Tumours found to penetrate into muscle and beyond the bladder wall contain losses on chromosomes 3, 4, 5, 6, 8, 11, 13 and 18. Halachmi et al.[119] has compared the probable (suggested) genetic events with pathological stage and has proposed a unified genetic pathway of bladder cancer progression (Figure 2.2).

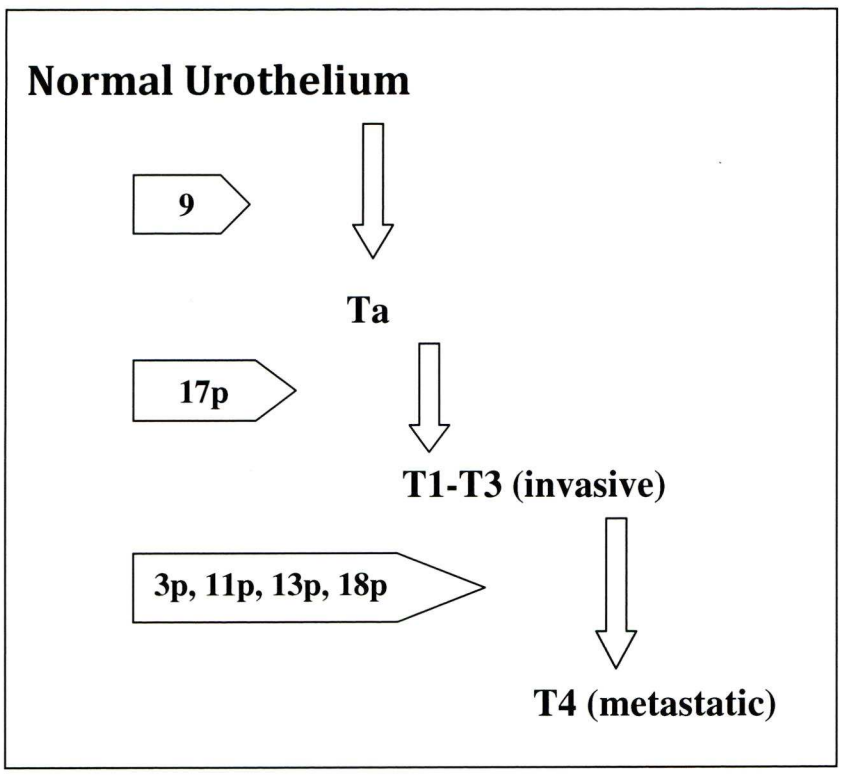


Figure 2.2 – Proposed association between progression of genetic events and evolution of the aggressive phenotype of bladder neoplasms

According to current understanding, mutations of tumor suppressor genes (e.g. p16, p53, pRb and PTEN) diminish control of proliferation and are permissive for accelerated growth. Thus, the dynamic equilibrium between cell proliferation and apoptosis becomes disturbed following deregulation of the cell-cycle, allowing events favouring tumourigenesis to dominate. The

rate of cell proliferation is an important factor contributing to initiation of a malignancy. Expression of Ki-67 protein is a widely accepted measure of this activity[120]. Apoptosis (programmed cell death) and regulation of cell division is recognized to be controlled by a number of proteins including Bcl-2, Bcl-X (L and S forms), Bax, Bad and Noxa, amongst others. In their non-mutated forms, tumour suppressor genes such as p53 and pRb inhibit cell proliferation and repress factors that might otherwise stimulate tumor development. Thereafter, malignancy is exploited by changes in cell-cell and cell-matrix adhesions of isoforms of CD44 and undetected by the reduced frequency of MHC antigens.

The genes currently considered to be some of the most important in the etiopathogenesis of bladder cancer, particularly those of transitional cell type, will be assessed in the remainder of this chapter. Wherever possible, their practical diagnostic utility, and limitations, are discussed and illustrated. While yet highly fragmented, a picture is now appearing of these genes, their complexity and their interactions, that promote and modulate individual malignant urothelial phenotypes, although the events initiating involvement of these genes remain stubbornly obscure.

2.2: Alterations in Gene Expression and Activity

Investigations to try to elucidate the early changes in bladder cancer and to understand the fundamental processes underlying the transition from initial genetic changes to invasion and metastasis has been a complex process.

Initial thoughts on the genetic pathways involved in bladder cancer were based largely on chromosomal (cytogenetic) mechanisms such as deletion or duplication of entire chromosome arms, or even whole chromosomes. The key events detected by this process showed the importance of the loss of the short arm of chromosome 9 (9p) and the subsequent loss of the short arm of chromosome 17 (17p). It is now known that the tumour suppressor gene p16 is located on chromosome 9p and p53 another key tumour suppressor is on chromosome 17p. Gains or amplification of chromosomal regions were associated with oncogene amplification such as that of the Ras genes. More recent knowledge of gene structure and function, gene mapping and sequencing culminating in the Human Genome Project has given much greater insights into the genetic changes that underlie tumour progression. It is now known that although chromosome loss is still one of the major components contributing to loss of function of tumour suppressor genes, there are other mechanisms that can contribute to loss (or gain) of function of genes that play critical roles in oncogenesis. It is now recognised that gene deletion or amplification may involve small microscopic deletions that are difficult to detect at the gross chromosomal level unless specific gene probes are used (examples of this are the tumour suppressor genes and HER2/neu). Genes may be inactivated or changed by mutation. Even single base changes can affect gene function, through mechanisms such as producing a stop codon, thus initiating premature termination of the messenger RNA produced or by affecting splice sites and thus splice variants. Genes can also be silenced by methylation, or their function altered by translocation so they are adjacent to other genes and control systems that

may result in their under or overproduction (this latter process is particularly important in the amplification of oncogenes). It is for these reasons that more detailed information about some of the major genes involved in bladder cancer progression aids understanding of the most fundamental processes at each stage of progression. From this, pathways of the processes underlying the major transitions in bladder cancer pathology have evolved from those of basic mechanisms utilising chromosomal changes alone, to those involving this rapidly expanding molecular genetic knowledge.

2.3: TUMOR SUPPRESSOR GENES

2.3.1: Gene: CDKN2A (Protein: p16)

Gene CDKN2A, also known P16, MTS1, TP16, CDKN2 or INK4a, maps on chromosome 9, at 9p21. The products of these genes localize in the nucleus where they have cyclin-dependent protein kinase inhibitor, tumor suppressor activities and are involved in cell-cycle arrest, cell-cycle check point, negative regulation of cell proliferation, regulation of CDK activity and oncogenesis functions.

The gene generates several transcript variants which differ in their first exons and utilize alternate polyadenylation sites. Its sequence produces, by alternative splicing, 12 different transcripts together encoding 11 different protein isoforms. There are seven likely alternative promotors and three

non-overlapping alternative last exons. Four alternatively spliced variants encoding distinct proteins have been reported, three of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript includes an alternate first exon located upstream of the remainder of the gene and contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the products of the other variants. This ARF product functions to stabilize p53 protein and is able to interact with, protein MDM1 responsible for p53 degradation. Although there are significant structural and functional differences, the CDK inhibitor isoforms and the ARF product encoded by this gene, are commonly involved in cell-cycle control at G1 through regulation of the activities of CDK4 and p53. This gene is frequently mutated or deleted in a wide variety of tumors, including bladder cancer, and is recognised to be an important tumor suppressor gene.

2.3.2: Gene: WAF1/CIP1 (Protein: p21)

The gene encoding protein CDKN1A is known as p21, CIP1, WAF1 or CDKN1 and maps on chromosome 6, at 6p21. The products localize in the nucleus and have potent cyclin-dependent protein kinase inhibitor, cell-cycle regulator activities involved in cell-cycle arrest. The gene is expressed at a very high level and produces, by alternative splicing, 13 different transcripts altogether encoding 12 different protein isoforms. The encoded protein binds to, and inhibits, the activity of cyclin-CDK2 or –CDK4

complexes, thus functioning as a regulator of cell-cycle progression at G_1. Expression of this gene is tightly controlled by tumor suppressor protein p53, through which this protein mediates the p53-dependent cell-cycle G1 phase arrest in response to a variety of stress stimuli. The WAF1/C1P1 gene contains at least two p53-responsive sites in its promoter region, responsible for transcription of p21 when bound to p53, thus suggesting protein p21 to be a potent downstream mediator of the anti-proliferative function of wild-type p53[121]. Conversely, a p53-independent pathway has been associated with p21 induction observed in various normal tissues during development or differentiation, in the absence of p53 activation as well as in tumour cells with mutated p53 [122-124].

Controversy persists concerning the prognostic utility of p21 expression in bladder cancers. Maintenance of p21 has been suggested to reduce the deleterious effects of altered p53 in TCC's[122]. Conversely, an associated p53 mutation appears to enhance chemosensitivity[125]. An inverse correlation has been suggested between p21/WAF1 immunoreactivity and tumour stage, grade, p53 accumulation and Ki-67 expression in muscle invasive tumours[126 127]. However, the value of immunohistochemically-derived data is now questionable (Figure 2.5). This study caveat applies not only to the WAF1/C1P1 gene, but also to the products of other genes with multiple-spliced products. Nevertheless, Quereshi *et al.* showed distinct prognostic groups with p21+, p53+ tumors associated with the best survival advantage and p21-, p53- tumors the worst[128]. Zlotta *et al.* reported increased p21 expression with the grade and stage of superficial tumors, attributing a

lower recurrence–free survival and a positive correlation with p53 but not with Ki-67[129]. Studies of p21 immunoexpression by Waldman et. al. reported better chemo-radiosensitivity in p21 negative bladder-cell lines raising the possibility of an antiapoptotic function [130].

2.3.3: Gene: TP53 (Protein: p53)

Gene TP53, also known as p53 or TRP53, maps on chromosome 17, at 17p13.1. The gene product, tumour protein (Tp53) is a nuclear protein that plays an essential role in regulating the cell-cycle, specifically during transition from G_0 to G_1. In normal cells, it is found in very low levels but in a variety of transformed cell lines it is expressed in high amounts, and is believed to contribute to neoplastic transformation and to malignancy. Tp53 is a DNA-binding protein containing DNA-binding and transcription activation domains. It is postulated that p53 activates expression of downstream genes that inhibit growth and/or invasion, thus functioning as a tumour suppressor. Alterations of the TP53 gene occur not only as somatic mutations in human malignancies, but also as germline mutations in some cancer-prone families.

Protein p53 regulates progression through the cell-cycle in G_1 and G_2 in response to DNA damage and to apoptosis which occurs following a range of DNA damaging agents[131]. In non-transformed cells, p53 causes arrest after G_1, allowing time for cell repair or apoptosis. The gene is inactivated

in up to 50% of all cancers including the bladder[132] and is the most frequently modified gene in bladder cancer. It is possibly the most important gene involved in the progression of this disease[133]. Although many different mutations are recognized to occur within the genome, mutated p53 usually differs by a single point mutation from the wild type resulting in an amino acid substitution. All such mutations occur in areas of the gene conserved by evolution. One particular region ('hotspot') comprises four codons in which more than 30% of mutations are known to occur. Inactivation of this gene inhibits activity of the normal protein resulting in a proliferative advantage to affected cells[134], causing them to become unstable. The retarded degradation and nuclear accumulation conferred upon abnormal p53 proteins is a reflection of elevated stability and self-aggregation of the mutated molecules[135]. Thus, mutant p53 products are more likely to be detected using immunohistochemical analysis.

The TP53 gene has been studied in urothelial carcinoma with mutations correlating with grade and stage[136 137], recurrence[138], progression[111], and survival[139]. Dahse *et al.* found TP53 abnormalities to be more frequent in high grade, muscle-invasive lesions and hypothesised that low grade bladder TCC's (Figure 2.4) proceed through another genomic pathway associated with the inactivation of CDK inhibitors p15, p16 and p21 [140]. Sarkis *et al.* showed a significant decrease in the survival rate of patients with muscle-invasive cancer and altered p53 function[141]. Bladder tumours with TP53 alterations exhibited more aggressive phenotypes and gene mutation rates

correlated with higher grading and/or staging together with muscle invasiveness[142].

2.3.4: Gene: RB1 (Retinoblastoma protein: pRb)

The RB1 gene maps on chromosome 13, at 13q14.2 where it consists of 27 exons[143]. It encodes a retinoblastoma-associated protein that has tumour suppressor activity. The gene sequence produces, by alternative splicing, 11 different transcripts altogether encoding 10 different protein isoforms. Retinoblastoma-like and retinoblastoma-associated proteins have regulatory functions in cell-cycle. The proteins form a complex with adenovirus E1A and SV40 large T antigen, and may bind and modulate the activities of certain cellular proteins with which T and E1A compete for pocket binding. These proteins may also act as tumour suppressors and are potent inhibitors of E2F-mediated trans-activation. Retinoblastoma protein regulates cell-cycle progression according to its own state of phosphorylation which reaches its peak at the beginning of S phase and is lowest after mitosis. Retinoblastoma protein pRB is a target for the enzymatic activity of cyclin-CDK complexes[144 145]. Phosphorylation of pRB occurs as cells progress from G_1 through late G_1, early S phase and G_2. The active underphosphorylated form of pRB exerts a negative regulatory effect on gene expression in G_0 to middle G_1 through complex formation with DNA-binding proteins[146]. Stimulation of quiescent cells with mitogens induces phosphorylation of pRb, whereas differentiation of the same cells

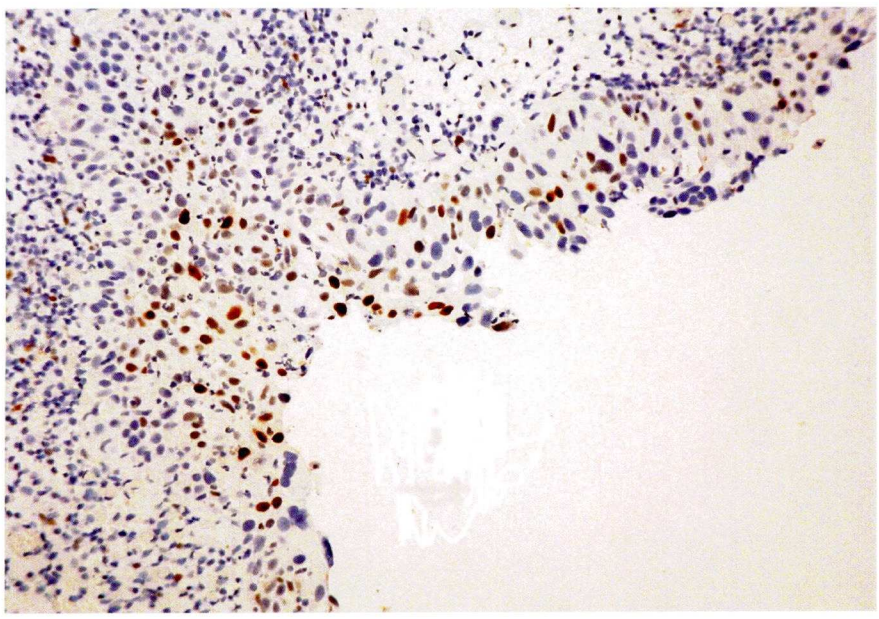


Figure 2.3: Heterogenous expression of p21 gene product by a flat urothelial lesion that is p53 mutation-positive and also PTEN downregulated

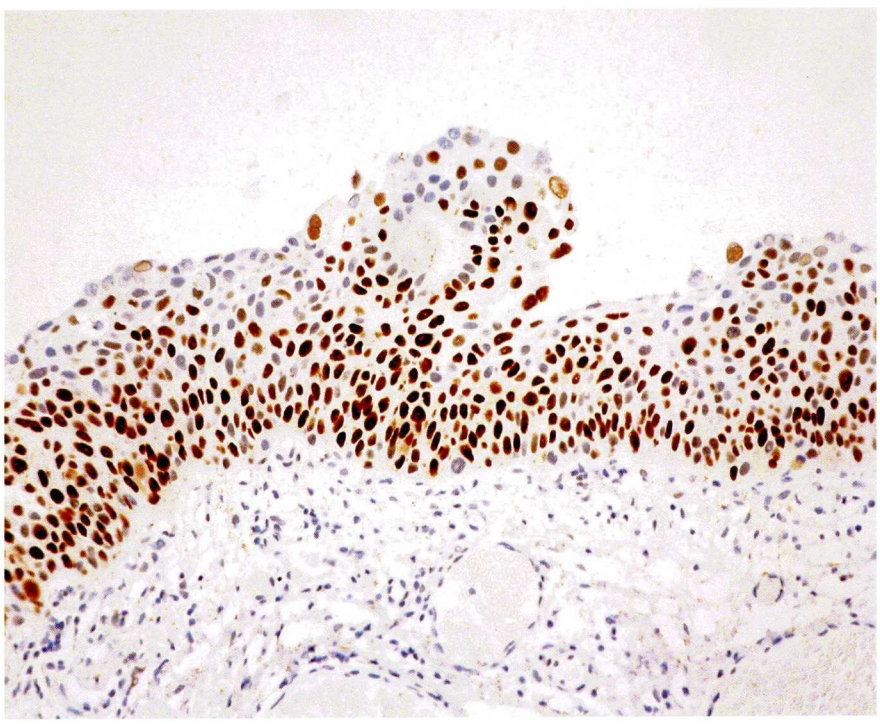


Figure 2.4: Early papillary urothelial carcinoma containing a p53 mutation.

Figure 2.5: Early papillary urothelial carcinoma containing Rb mutation in which the urothelial cells are morphologically unremarkable

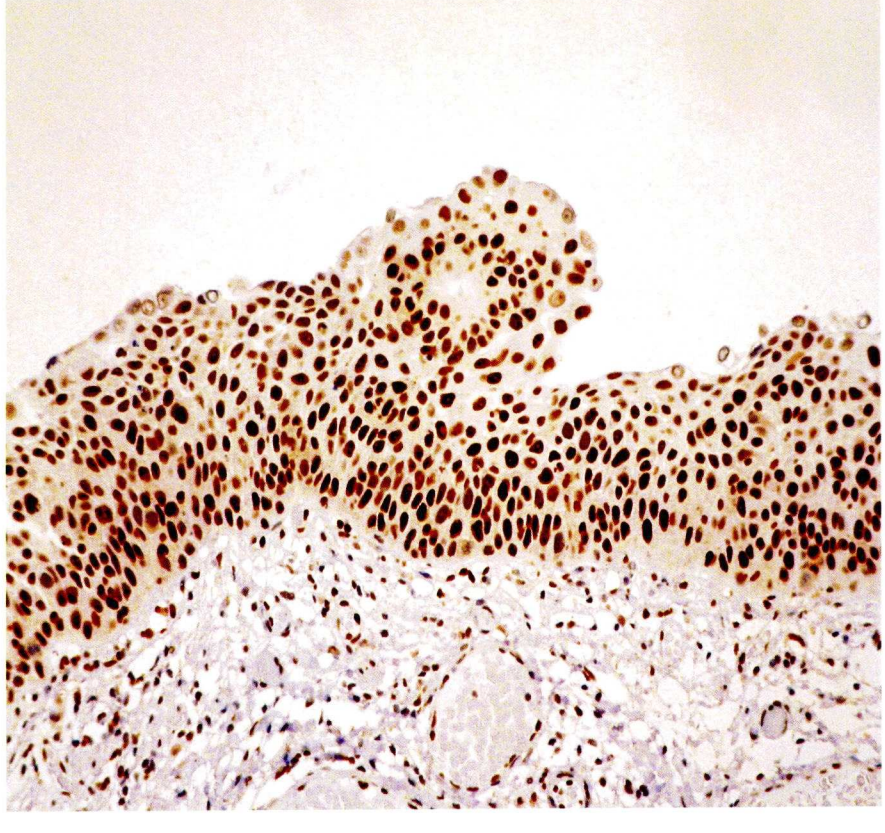


Figure 2.6: Early papillary urothelial carcinoma-Rb & p53 positive

induces hypophosphorylation. It is the hypophosphorylated form that suppresses cell proliferation[147]. Mutations to the Rb gene can occur through a number of different mechanisms, all causing inactivation of pRb. Large scale deletions account for 30% of mutations, the remaining 70% being caused by splicing errors, point mutations and small deletions in the promoter region.

Mutagenic deactivation of RB produces a spectrum of altered expression patterns, from undetectable pRB levels (Figure 2.5) to heterogeneous cytoplasmic localisation of truncated pRB products that have lost their nuclear localisation signal[148 149]. Thus, in any bladder carcinoma, the precise levels of pRb are not static but are in constant flux (Figure 2.6). Furthermore, these pRb proteins are subject to splice variation, making single-agent detection highly unreliable. Cordon-Cardo *et al.* found significant associations between undetectable pRB and tumor stage, grade, disease progression and reduced survival[150]. Altered Rb expression, identified immunohistochemically, was associated with a high rate of recurrence and poor outcome in T_1 tumours[151]. Mutated expression of the Rb gene found in association with muscle invasive tumors suggests a functional importance during acquisition of an invasive capability[152].

There is significant association between p53 nuclear over-expression and undetectable pRB[150], including a synergistic carcinogenic effect between mutated Rb and p53 such that patients with both alterations manifest an advanced tumor grade, marked progression and decreased survival. TP53

appears to target p21/WAF1, which encodes a CDK inhibitor[121]; pRB being a substrate for cyclin-CDK complexes[144 145].

2.3.5: Gene: PTEN

The gene PTEN is also known as BZS, MHAM, TEP1, MMAC1 or PTEN1 maps on chromosome 10, at 10q23.3. Its sequence produces, by alternative splicing, 9 different transcripts together encoding 9 different protein isoforms. It encodes a phosphatase tensin homolog family member. The products localize in cytoplasm, have tumor suppressor, protein tyrosine phosphatase activities and are involved in regulation of CDK activity, development, cell proliferations and protein amino acid dephosphorylation.

PTEN is a major tumour suppressor gene frequently mutated or deleted in a wide range of tumours. It encodes a dual-specific phosphatase which, when non-mutated and active, can restrain the capacity of tumour cells to invade through a basement membrane matrix (Figure 2.7). PTEN over expression leads to changes in cell adhesion, spreading and inhibits cell migration[153]. Studies have found involvement of PTEN protein in the regulation of cell migration and invasion with its loss being significant in late stages of tumour progression[153 154]. The response of PTEN in bladder neoplasia is complex and its role remains to be elucidated in individual cancers. Immunohistochemical studies confirm the protein to be modulated in an individual and idiosyncratic manner. In non neoplastic transitional epithelium, PTEN is strongly expressed by many basal cells.

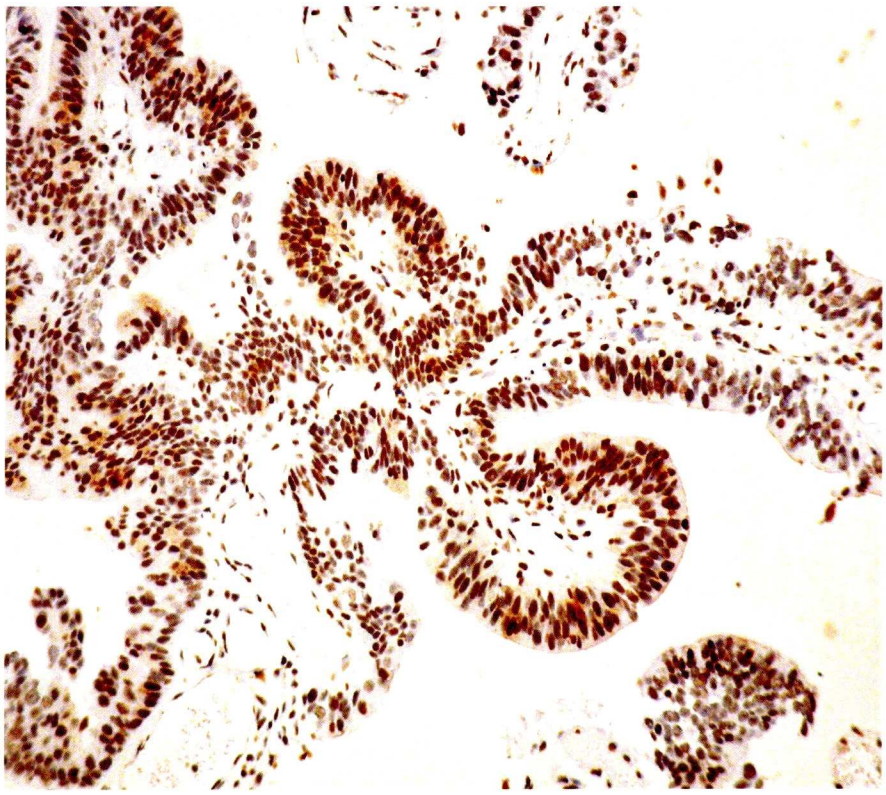


Figure 2.7: Early papillary urothelial carcinoma-strongly PTEN positive

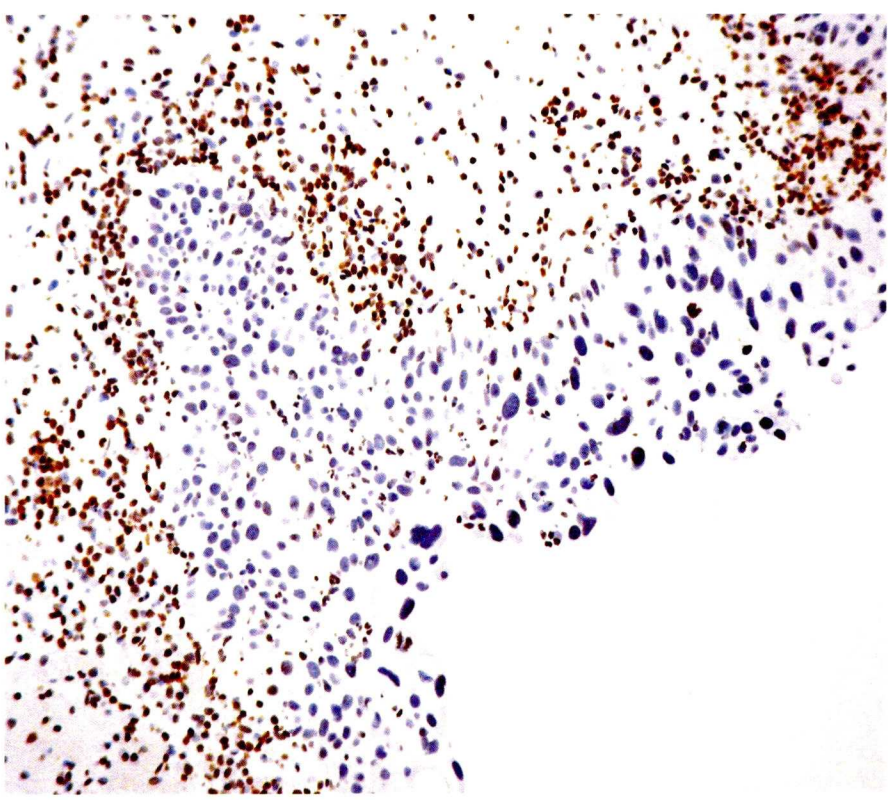


Figure 2.8: Early flat neoplasia-PTEN negative; p21 positive

However, in some neoplasms, early PTEN expression becomes diminished (Figure 2.8) only to recur at high intensity as the tumour becomes invasive. Unfortunately, immunohistochemistry is insufficiently sensitive to discriminate whether there is a quantitative or qualitative change in expression of the products of this gene.

2.4: ONCOGENES

Oncogenic gene sequences encode proteins that have normal function and are described as "proto-oncogenes". However, when the gene is mutated ("oncogene"), the abnormal protein product becomes a strong independent oncogenic stimulus that operates outwith normal cellular regulatory mechanisms.

2.4.1: RAS genes

The ras gene maps on the X chromosome where it encodes ras-related protein Rab-6B. It is expressed at low level in normal cells and produces by, alternative splicing, two different transcripts together encoding two different protein isoforms. There are two probable alternative promoters. The transcripts appear to differ by truncation of the N-terminus, because an internal intron is not always spliced-out.

The ras branch of the ras super family consists of small GTPases most closely related to ras and include the r-ras, Rap, Ral, Rheb, Rin and Rit proteins. Although understanding ras signalling and biology is now considerable, recent observations suggest that the function of these proteins is more complex than previous believed. First, the different ras proteins may not be functionally identical. Second, ras protein function involves interactive cross-talk with their close relatives.

The mammalian ras gene family consists of the Harvey and Kirsten ras genes (c-Hras1 and c-Kras2), an inactive pseudogene of each (c-Hras2 and c-Kras1) and the N-ras gene. These proteins differ significantly only in their C-terminal 40 amino acids. These ras genes have GTP/GDP binding and GTPase activity, and their normal function may be as G-like regulatory proteins involved in the normal control of cell growth. Point mutations in the three members of the RAS proto-oncogene family (H-ras, K-ras, N-ras) are common in human cancers[155] and suspected in the development and progression of many human bladder cancers. A high incidence of both H-ras and K-ras mutations at codon 12 have been reported in histochemically-confirmed bladder cancers[155 156]. In these tumours, Przybojewska et al. found frequent N-ras gene mutation at codon 61 associated with H-ras mutations[155]. Fontana et al. demonstrated a significant relationship between enhanced c-ras oncogene expression and early recurrence in superficial bladder cancer[157].

2.4.1.1: Gene: H-RAS

The Harvey ras gene (H-RAS) also known as HRAS1 or RAS111, maps on chromosome 11, at 11p15.5. The gene encodes transforming protein products that have GTP binding properties, GTPase activities and are involved in small GTPase-mediated signal transduction. The gene is expressed at very high level and its sequence produces, by alternative splicing, 9 different transcripts together encoding 5 different protein isoforms.

2.4.1.2: Gene: K-RAS2

The Kirsten ras gene (K-RAS2) also known as RASK2, KI-RAS, C-K-RAS, K-RAS2A or K-RAS2B, maps on chromosome 12, at 12p12.1. It encodes transforming GTPase protein products that have GTPase activity and are involved in small GTPase-mediated signal transduction. The gene sequence produces, by alternative splicing, 3 different transcripts altogether encoding 3 different protein isoforms. Single amino acid substitutions are responsible for activating mutations. Alternative splicing leads to variants encoding two isoforms that differ in the C-terminal region.

2.4.1.3: Gene: N-RAS

The N-RAS gene also known as N-ras or NRAS1, maps on chromosome 1, at 1p13.2. It encodes transforming protein products predicted to have small GTP binding ability and GTPase activities, and to be involved in small GTPase-mediated signal transduction. Mutations which change amino acid residues 12, 13 or 61 activate the potential of N-ras to transform cells in tissue culture and are implicated in a variety of human tumours.

2.4.2: Gene: c-ERBB-2

ERBB2 gene also known as NEU, NGL, HER2, TKR1 or HER-2, maps on chromosome 17. at 17q21.1[158 159]. The protein product is a transmembrane glycoprotein within the receptor protein-tyrosine kinase family[160]. Specifically, the protein exhibits Neu/ErbB-2 receptor activity in addition to protein kinase, transmembrane receptor protein tyrosine kinase, epidermal growth factor receptor and ATP binding activities. The protein products of the gene localize in membrane and are involved in protein amino acid phosphorylation and transmembrane receptor protein tyrosine kinase signalling pathways. The gene sequence produces, by alternative splicing, 19 different transcripts, together encoding 19 different protein isoforms.

ErbB-2 (also known as HER-2/neu) is a proto-oncogene implicated in a number of tumors[161]. Some of these proto-oncogenes are related to growth factors or to growth factor receptors (such as c-sis, c-fms, c-erbB-1 and c-

erbB-2)[162-164]. Over expression and amplification in bladder cancer (Figure 2.9) correlates with tumor grade, re-occurrence and disease progression[165] [166]. Several reports indicate a significant relationship between erbB-2, p53 and increased cell proliferation[167] [168], with these oncoproteins rarely expressed in superficial tumors[167] [169-171]. ErbB-2 over expression appears to precede disease progression suggesting prognostic value[166].

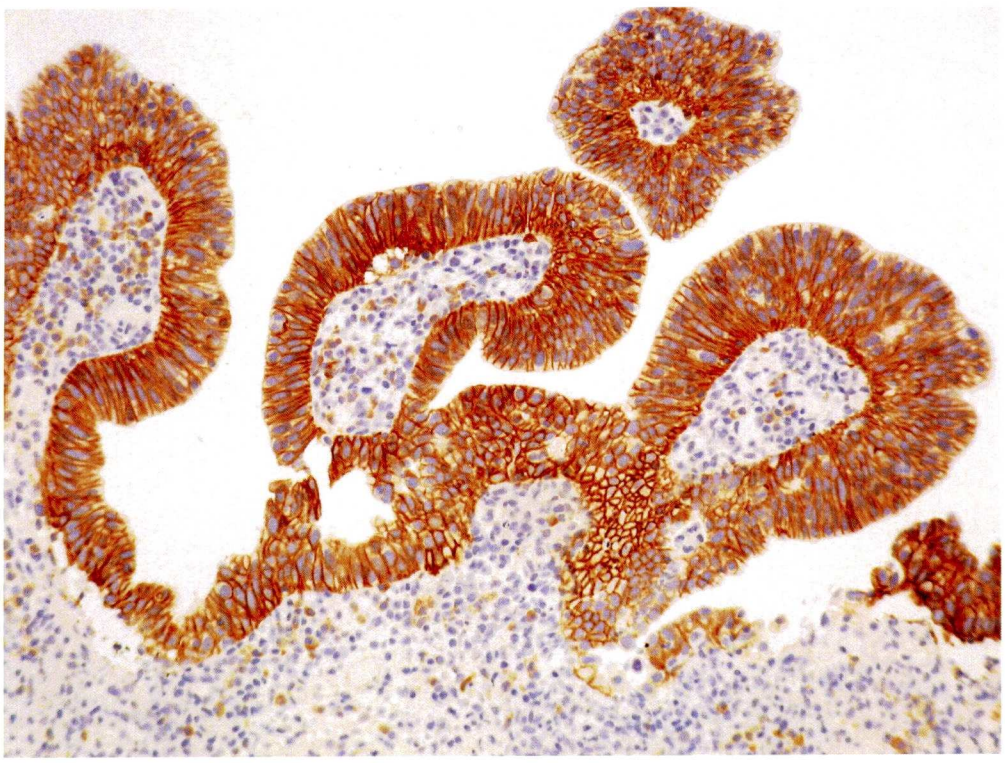


Figure 2.9: Morphologically dysplastic urothelium- strong HER2/neu

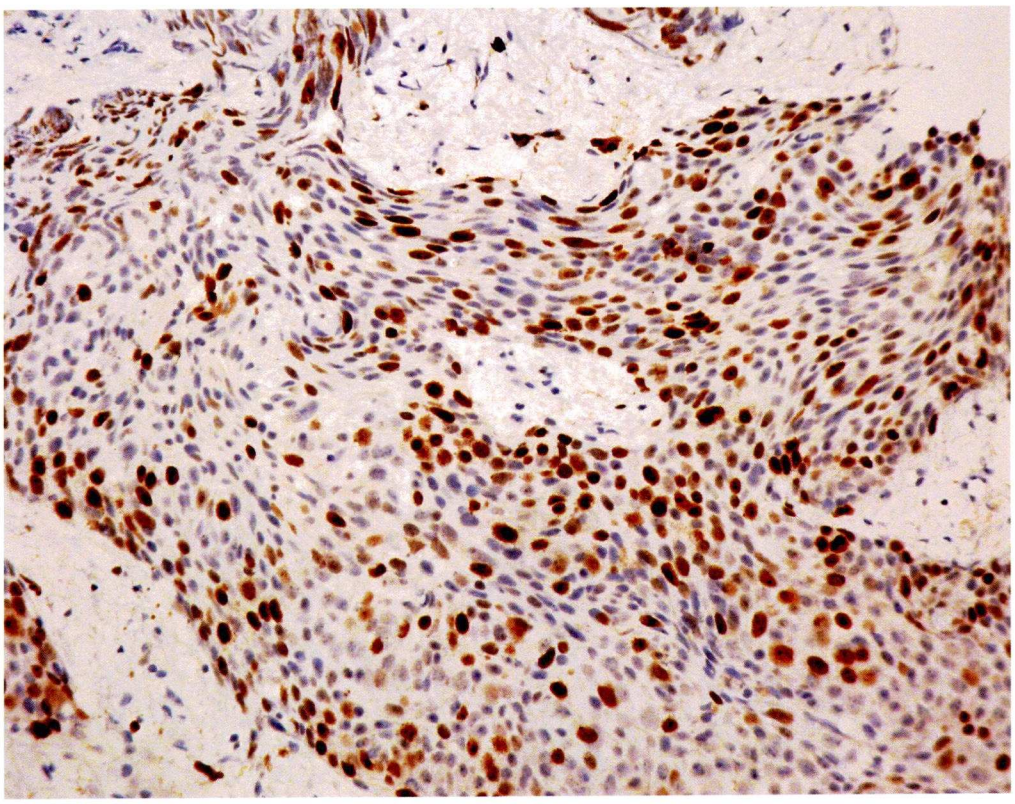


Figure 2.10: G3pT1 carcinoma- strongly expresses nuclear Ki-67 protein

2.5: TUMOR MODULATING GENES

2.5.1: Protein: Ki-67

This is the product of gene MK167, also known as KIA, which maps on chromosome 10, at 10q25. It is expressed at very high level in all cells. The gene produces, by alternative splicing, three types of transcripts predicted to encode three distinct proteins. Protein Ki-67, detected by immunohistochemically murine monoclonal antibody MCB1, is a good indicator of proliferation in a variety of tumours, including bladder[172 173]. This proliferation-associated nuclear antigen is expressed in all cells in G1, S and G2M phases but not by cells in G0, thus allowing assessment of the entire proliferating cell pool[174]. Ki-67 expression studies have shown a strong overall correlation with tumour stage and grade[175]. High grade TCC's (Figure 2.10) tend to have a higher Ki-67 index than low-grade tumours, although this is not a universal or invariable relationship. Studies have also shown a correlation with tumour aggressiveness, both recurrence and progression to invasive disease[176] and metastasis. Ki-67 index can help distinguish Grade 2 tumours with a favourable outcome from clinically unfavourable tumours of identical morphology[177]. A Ki-67 labelling index over 20% predicts that morphologically well-differentiated tumours of pathological stage pTa/pT_1 will recur within a year of diagnosis[178].

2.6: CYP2B6 encoding cytochrome P450

Gene CYP2B6, also known as CPB6, IIB1, P450, or CYPIIB6 maps on chromosome 19, at 19q13.2. The gene encodes a member (P-450 2B-BX) of the cytochrome P450 superfamily of enzymes, is moderately expressed and produces 2 different protein isoforms by alternative splicing. There are 2 probable alternative promoters, although these have yet to be confirmed by functional studies. The transcripts appear to differ by truncation of the N-terminus, presence or absence of a cassette exon by alternative splicing the gene produces 2 types of transcripts, predicted to encode 2 distinct proteins. It contains 9 confirmed introns, 4 of which are alternative. The products are involved in electron transport.

Cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism, in the synthesis of cholesterol, steroids and other lipids, and hence are indirectly involved in maintaining cellular homeostasis. This protein localizes to the endoplasmic reticulum and its expression is induced by phenobarbital. The enzyme is known to metabolize some xenobiotics, such as the anti-cancer drugs cyclophosphamide and ifosphamide. Transcript variants for this gene have been described. However, it has not been confirmed whether these transcripts are produced by this gene or by closely related pseudogene, CYP2B7. Both the gene and the pseudogene are located in the middle of a CYP2A pseudogene found on chromosome 19q within a large cluster of cytochrome P450 genes from the CYP2A, CYP2B and CYP2F subfamilies.

The cytochrome P450 motif is found in 2 isoforms from this gene. 62 other genes in the database also contain this motif. The cytochrome P450 enzymes usually act as terminal oxidases in multicomponent electron transfer chains. The significance of this observation is self-evident with respect to the production of monoclonal antibodies to specific proteins and to possible overlapping functions of apparently dissimilar proteins.

2.7: Gene: FGFR3 encoding fibroblast growth factor receptor 3

Gene FGFR3, also known as ACH, CEK2, JTK4 or HSFGFR3EX maps on chromosome 4, at 4p16.3. It encodes a growth factor receptor with ATP binding and protein kinase activities. It is involved in protein amino acid phosphorylation. The gene is expressed at high level and produces, by alternative splicing, 7 different transcripts altogether encoding 7 different protein isoforms. There are 2 probable alternative promotors and 2 non overlapping alternative last exons. The transcripts appear to differ by truncation of the N-terminus, truncation of the C-terminus, presence or absence of 4 cassette exons, common exons with different boundaries, because an internal intron is not always spliced out.

The protein encoded by this gene is a member of the fibroblast growth factor receptor family, where amino acid sequence is highly conserved between members and throughout evolution. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-

length representative protein comprises an extracellular region containing three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, initiating a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds acidic and basic fibroblast growth hormone.

2.8.1: Gene: ESR1 encoding estrogen receptor 1 (ER alpha)

Gene ESR1, also known as ER, ESR, Era, ESRA or NR3A1 maps on chromosome 6, at 6q25.1. It encodes an estrogen receptor localized in nucleus with DNA binding, transcription factor, steroid hormone receptor and steroid binding activities. The products are involved in the regulation of transcription. Estrogen receptor (ESR) is a ligand-activated transcription factor composed of several domains that are important for hormone binding, DNA binding, and activation of transcription. It is expressed at high level in many cells and produces, by alternative splicing, 11 different transcripts together encoding 11 different protein isoforms. There are 5 probable alternative promoters and 5 non overlapping alternative last exons. The transcripts appear to differ by truncation of the N-terminus, truncation of the C-terminus, presence or absence of 5 cassette exons, common exons with different boundaries.

The ligand-binding domain of this nuclear hormone receptor motif is found in 7 isoforms from this gene. At least 47 other genes are known to contain this motif. The steroid hormones and their receptors are involved in the regulation of eukaryotic gene expression and affect cellular proliferation and differentiation in target tissues. In the absence of ligand, steroid hormone receptors are thought to be weakly associated with nuclear components. Hormone binding greatly increases receptor affinity. The hormone-receptor complex appears to recognize discrete DNA sequences upstream of transcriptional start sites. The estrogen receptor motif is found in 5 isoforms from this gene. No other gene in the database contains this motif.

2.8.2: Gene ESR2 encoding estrogen receptor 2 (ER beta)

Gene ESR2, also known as Erb, ESRB, NR3A2, ER-BETA or ESR-BETA maps on chromosome 14, at 14q. It encodes estrogen receptor beta that is well expressed in many different cell types. The gene produces, by alternative splicing, 9 types of transcripts, predicted to encode 7 distinct protein isoforms. It contains 28 confirmed introns, 27 of which are alternative. There are 6 probable alternative promotors and 4 non overlapping alternative last exons. The transcripts appear to differ by truncation of the N-terminus, truncation of the C-terminus, and the presence or absence of 8 cassette exons. The products have a wide range of properties including steroid binding, transcription co-activator, transcription factor,

receptor antagonist, and estrogen receptor activities. The gene products are involved in regulation of transcription, DNA-dependent, signal transduction, cell-cell signalling, negative regulation of cell growth, estrogen receptor signalling pathway and localize in the nucleus. Estrogen receptor-beta (ESR2) is a member of the super family of nuclear receptors, which transduce extracellular signals into transcriptional responses. The gene is antisense to gene SYNE2. The possibility that its mRNAs may be present and stable only in the absence of matching mRNAs from the gene on the other strand and vice versa is a distinct possibility.

2.9: Gene: Epidermal growth factor receptor (EGF-R)

The EGF-R gene, also known as ERBB or ERBB1, maps on chromosome 7 at 7p12 and encodes an epidermal growth factor receptor with protein kinase, transmembrane receptor protein tyrosine kinase and ATP binding activities. The receptor is involved in protein amino acid phosphorylation, transmembrane receptor protein tyrosine kinase signalling and localizes in membrane. The gene is expressed at very high level and produces, by alternative splicing, 10 different transcripts altogether encoding 10 different protein isoforms. EGF-R is a 170 kDa protein product of a proto-oncogene with three domains that are extracellular, transmembrane and intracellular. Activation of EGF-R by ligands such as epidermal growth factor, transforming growth factor-α, betacellulin and amphiregulin results in stimulation of the ras/raf, phophatidylinositol-3-kinase and protein kinase C

pathways culminating in increased nuclear transcription and cellular proliferation[179] as well as increased angiogenesis and reduced apoptosis.

EGF-R immuno-expression, normally confined to the basal cell layer of urinary bladder epithelium[180 181], is reported to be over-expressed in the entire urothelium involved in bladder TCC[169 171 182-184]. Over-expression of EGF-R correlates with a shorter interval to recurrence, higher recurrence rate and an increased rate of progression in patients with superficial bladder cancer but not in patients with muscle invasive disease[185]. Its expression suggests that the phenotype of an individual bladder cancer in not static but evolves with the stage of disease progression. Stein et al.[185] suggested that increased expression of EGF-R in histologically normal urothelium or the presence of high EGF-R levels in normal urothelium distant to a tumour site[181] are early events in bladder tumourigenesis.

2.10: Heat Shock Proteins

There are two known genes encoding homeostatic regulatory proteins in this family: The gene known as HSPB1, also as HPS27, HSP28 or HSP25 maps on chromosome 7, at 7q11.23. The protein products have heat shock protein activity and are involved in regulation of translational initiation and localize in the cytoplasm. The gene is expressed at a very high level and produces, by alternative splicing, 16 different transcripts altogether encoding 13 different protein isoforms. There are 2 probable alternative promoters.

The gene known as HSPB2 also as MKBP, HSP27, HSP72, HS.78846, FLJ25219 or MGC14839 maps on chromosome 11, at 11q22-q23. The products localize within the cytosol, have enzyme activator, protein binding, heat shock protein activities and are involved in response to cellular "stress". The gene is expressed at high level and produces, by alternative splicing, 6 different transcripts altogether encoding 6 different protein isoforms. Its regulation may use antisense and coregulation with neighbouring genes organized in an operon-like structure.

Heat shock protein (hsp) modulation is an essential cellular defence mechanism found in both prokaryotic and eukaryotic organisms as a response to a variety of stress insults. Hsp-27, -60, -70 and –90, present in all living cells, play key cytoprotective roles as molecular chaperones of other proteins. They participate in developing cellular resistance and intracellular protein stabilisation[186 187]. By inhibiting apoptosome formation,

hsp-70 inhibits the receptor or mitochondrial pathways hampering the caspase cascade that leads to apoptosis[188]. Hsp-70 interacts with p53 protein, stabilising the mutant form, thus permitting cell proliferation that is thereafter unregulated by this pathway[189]. Hsp's act as antigen presenters and play an important role in antibody assembly. Over expression of hsp's has been associated with poor prognosis but may increase immunogenecity in some tumours, potentiating immune response and improved overall survival[190]. In superficial bladder cancers with a propensity to recur[191], studies have shown that hsp over-expression may be an important prognosticator of BCG response. The universal expression of hsp-27 in normal urothelium makes this a powerful immunohistochemical reagent with which to detect early invasive disease (Figure 2.11). However, some transitional cell carcinomas express hsp-27 only at very low level, although immune-reactivity may be dependent upon the expression of individual splice-variants and the reactivity of particular monoclonal antibodies with these variants. Nevertheless, relative absence of this protein from these tumors appears to be a strong (but hitherto unconfirmed) indicator of their potential aggressiveness.

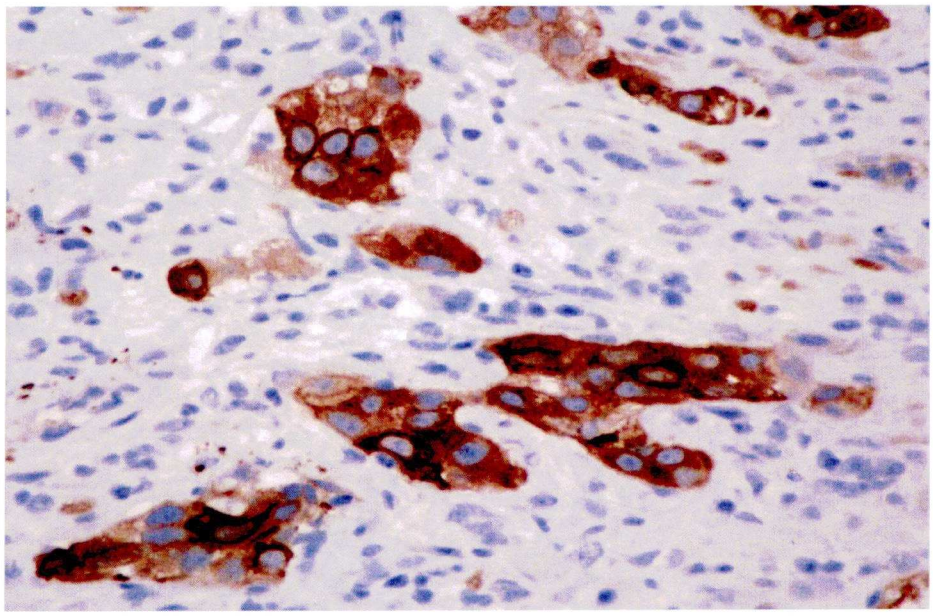


Figure 2.11: Small clusters of Invasive tumour within submucosa-strong HSP-27 expression. Invaluable marker in discriminating pTa from pT1

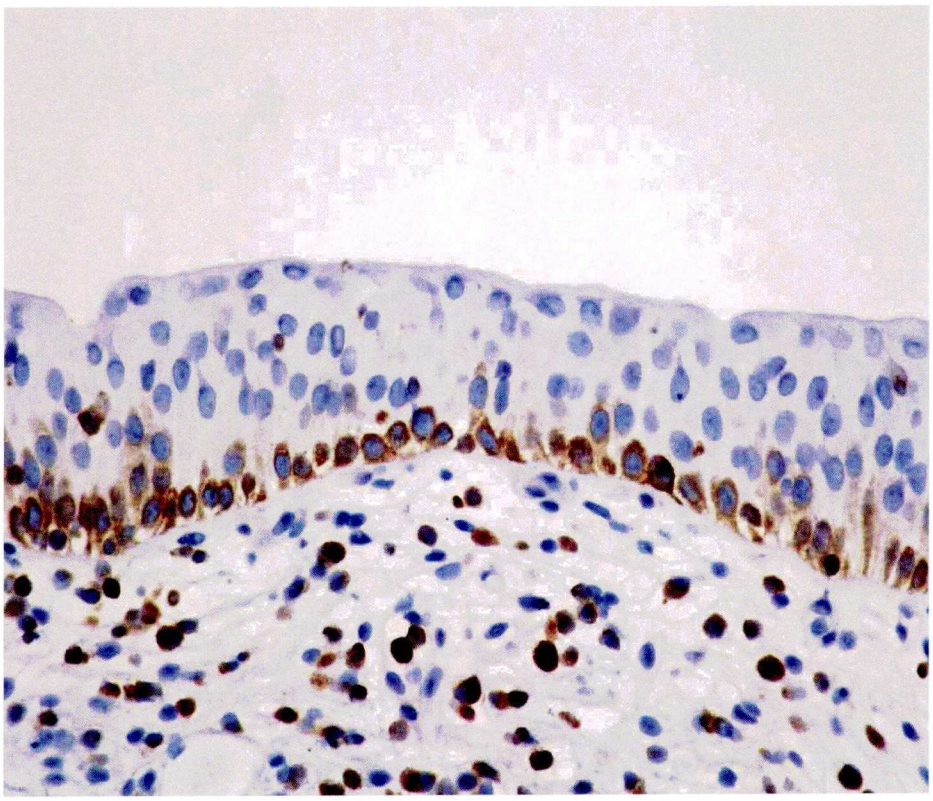


Figure 2.12: Normal urothelium with bcl-2 positive in basal layer. Loss of bcl-2 occurs early in neoplasia

2.11: Apoptotic Modulators

2.11.1: Gene: Bcl-2

The chromosomal localization and sequence of the bcl-2 gene has not yet been mapped. However, the principal product of the bcl-2 gene is a ~25-26 kDa integral membrane protein, mainly located in the outer mitochondrial membrane and to a lesser extent in the endoplasmic reticulum and nuclear envelope. It is known that presence of the bcl-2 protein promotes cell survival, even when the rate of cell proliferation is not high, thus providing a growth advantage that eventually may lead to neoplastic transformation[192]. This may be of particular importance in the normal bladder where the proliferation index of normal urothelium is extremely low. Krajewski *et al.* suspected bcl-2 to influence the activity of Ca^{2+}- dependant enzymes involved in apoptosis by regulating the activity of Ca^{2+} pumps or channels in the ER, mitochondrial and nuclear membranes[193]. Nakopoulou et al. [194]observed an increased bcl-2 expression in low-grade TCC's and in superficial rather than invasive TCC's. Conversely, high grade, aggressive and advanced tumors tend to show non-basal bcl-2 over-expression[195-198]. This finding suggested a deregulation of mechanisms controlling bcl-2 expression. Prognostic significance of bcl-2 expression is controversial with Atug *et al.*[196] and Kirsh *et al.*[199] concluding an unfavourable outcome, refuted by Li *et al.* [198 200]and Lipponen *et al.*[197]. The common expression of bcl-2 in normal (Figure 2.12) and dysplastic urothelium but its loss from low grade TCC suggests that bcl-2 expression may be an early event in TCC

tumorigenesis[201]. Also, a reciprocal relationship between bcl-2 and p53 has been reported with presence of p53 implying greater probability of invasion[202-205]. These earlier findings are at some variance with respect to current findings from this laboratory.

Apoptosis (programmed cell death) plays an important role in physiological processes such as embryogenesis, organ development and cell proliferation. It is also an important function in pathological processes including autoimmune disease and cancer development[206]. Bcl-2 belongs to a family containing both pro- and anti-apoptopic genes[207]. Bcl-2 inhibits apoptosis induced by a number of stimuli such as radiation, chemotherapeutic agents and growth factor deprivation. It is unusual in that its function assists neoplastic growth by prolonging cell survival through inhibition of apoptosis rather than accelerating cell proliferation, thus enhancing the metastatic potential of a tumor[206]. Bcl-X_L, from the same family, is also an anti-apoptopic gene which displays distinct patterns of expression to Bcl-2 in TCC's, suggesting that the two proteins regulate different cellular functions at specific stages of cell differentiation[199].

2.11.2: Gene: Bcl-X

This gene BCK2L1, also known as BCLX, BCL2L, Bcl-X, bcl-xL, bcl-xS or BCL-XXL/S, maps on chromosome 20, at 20q11.21. The gene sequence produces, by alternative splicing, 21 different transcripts altogether encoding 9 different protein isoforms. There are 4 probable alternative promoters. The transcripts appear to differ by truncation of the N-terminus, presence or absence of 3 cassette exons, common exons with different boundaries. As a result of alternate splicing the gene encodes two functionally different proteins. The longer Bcl-X_L is anti-apoptopic while the shorter Bcl-X_S facilitates apoptosis by interfering with the action of Bcl-2[199 207 208]. The proteins encoded by this gene are located at the outer mitochondrial membrane, and have been shown to regulate outer mitochondrial membrane channel (VDAC) opening. VDAC regulates mitochondrial membrane potential, and thus controls the production of reactive oxygen species and release of cytochrome C by mitochondria, both of which are the potent inducers of cell apoptosis. Two alternatively spliced transcript variants, which encode distinct isoforms, have been reported. These homologues of the bcl-2 gene family have been demonstrated to show a high degree of over expression in TCC but only moderately in normal urothelium[208]. Kirsh *et al.* found that immunohistochemical staining of bladder cancer cell lines reflected a comparatively higher Bcl-X_L expression than Bcl-X_S[199].

In this laboratory, evidence from immunohistochemical studies is at variance with some findings reported elsewhere. Previously, strong expression of Bcl-2 in the basal cells of normal transitional epithelium (Figure 2.12) becomes attenuated as an early event in bladder neoplasia, even when the architecture is not overtly dysplastic. Surprisingly, expression of this protein occurs infrequently in established bladder cancers – whether in situ or invasive. This consistent lack of immunohistochemical staining may be due to a number of factors including the specificity of the particular antibody employed and possible splice-variants of the proteins expressed in bladder neoplasia. Nevertheless, there is a distinct reciprocal correlation between diminished expression of Bcl-2 and enhanced expression of HER2/neu suggesting that the observed loss of identified splice-variant of the protein is a real phenomenon.

2.12: Gene: CD44 ADHESION MOLECULE

Gene CD44, also known as IN, MC56, MDU2, MDU3, MIC3, Pgp1 or CD44R, maps on chromosome 11, at 11p13 and contains at least 20 exons[106 209,210]. It encodes cell surface glycoprotein CD44 that has collagen binding, hyaluronic acid binding and cell adhesion receptor activities. The gene is expressed at very high level and its sequence produces, by alternative splicing, 21 different transcripts altogether encoding 21 different protein isoforms.

The term "CD44" refers to a family of widely distributed cell surface (cluster differentiation) glycoproteins with an array of functions and many isoforms. The inappropriate and overabundant expression of this gene is of potential value in tumor diagnosis and in prognostic evaluation[210 211] as well as in further understanding of adhesion aspects of tumor biology. A correlation has been identified in the altered quantity and distribution of CD44 variant isoforms (CD44v) and in the progression of a number of different tumours[212]. CD44v6 isoform expression is reduced in poorly differentiated, invasive urothelial cancer cell lines when compared to well- or moderately-differentiated non-invasive tumors[213-215]. In bladder cancer it has been reported that overexpression of the CD44 locus is seen early in malignancy, this progressively diminishes as the tumor invades deeper into the bladder wall and is linked to loss of function of cell-cell cohesion, detachment of the basement membrane and infiltration of the surrounding

muscle. The pattern of CD44 expression confirms that the environment surrounding a tumor cell affects its regulation [106].

Correlation between CD44 expression and prognosis has been reported with strong expression related to high survival probability in muscle invasive tumors[213]. Toma et al. found that focal loss of immunostaining against CD44v3 and -v6 was a significant factor in identifying patients with non-muscle invasive urothelial carcinoma at high risk of recurrence[216]. Sugino *et al.* found that detection of CD44 overexpression was of importance not only in detecting early bladder cancer but also in evaluating borderline lesions[106].

2.13: Gene: Major Histocompatibility Complex (MHC) Antigens

The MHC superfamily of molecules are hetero-dimeric cell surface receptors that function to present antigen peptide fragments to T cells responsible for cell-mediated immune responses. MHC molecules can be subdivided into two groups on the basis of their structure and function: Class I molecules present intracellular antigen peptide fragments (~10 amino acids) on the surface of the host cells to cytotoxic T cells; Class II molecules present exogenously derived antigenic peptides (~15 amino acids) to helper T cells.

MHC molecules comprise two chains. In Class I the alpha chain is composed of three extracellular domains: a transmembrane region and a

cytoplasmic tail. The beta chain (beta-2-microglobulin) is composed of a single extracellular domain. In Class II, both the alpha and the beta chains are composed of two extracellular domains, a transmembrane region and a cytoplasmic tail. It is known that the Ig constant chain domains and a single extracellular domain in each type of MHC chains are related. These homologous domains are approximately one hundred amino acids long and include a conserved intradomain disulfide bond. Members of the immunoglobulin superfamily are found in hundreds of proteins of different functions. The existences of such regions of structural homology predict an identification system of adequately high sensitivity to distinguish these similar proteins. Antibodies are unlikely to provide sufficient discrimination. Such a system requires the precision of a molecular biological hybridization technique.

2.13.1: Gene: HLA-A (Class I)

The HLA-A gene, also known as HLA-A.1 or HLA-J maps on chromosome 6, at 6p21.3. It encodes protein products that localize in membrane and are involved in immune response. Its sequence produces, by alternative splicing, 34 different transcripts altogether encoding 34 different protein isoforms. There are 10 probable alternative promoters and 7 non overlapping alternative last exons. The transcripts appear to differ by truncation of the N-terminus, truncation of the C-terminus, presence or absence of 37 cassette exons or common exons with different boundaries.

HLA-A belongs to the MHC Class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane. Class I molecules play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. They are expressed in nearly all cells. The heavy chain is approximately 45 kDa and its gene contains 8 exons. Exon one encodes the leader peptide, exons 2 and 3 encode the alpha-1 and alpha-2 domains, which both bind the peptide, exon 4 encodes the alpha3 domain, exon 5 encodes the transmembrane region, and exons 6 and 7 encode the cytoplasmic tail. Polymorphisms within exon 2 and exon 3 are responsible for the peptide binding specificity of each class I molecule.

2.14: Gene: B_2M, protein beta-2 microglobulin

This gene B_2M, also known as RNF36 or Trif, maps on chromosome 15, at 15q21-q22.2. The gene is expressed at very high level in a wide range of epithelial and reticuloendothelial cells. The sequence produces, by alternative splicing, 40 different transcripts altogether encoding 35 different protein isoforms, all of which are involved in the presentation of endogenous antigen. There are 7 probable alternative promoters and 5 non overlapping alternative last exons. The transcripts appear to differ by

truncation of the N-terminus, truncation of the C-terminus, presence or absence of 22 cassette exons or common exons with different boundaries.

The MHC plays a vital role in the interaction between a number of cells including those of the immune system. Reduction of Class I antigens has been reported in a number of tumours. In bladder cancer, this reduction is associated with poor survival[217]. These antigens are the main target for natural killer cells (NK) which destroy cells displaying foreign genetic material after cell interactions such as those infected by viruses or which have undergone malignant transformation. In situations where expression of MHC Class II molecules become down-regulated, or "masked" by sialic acid, affected cells appear to remain immunologically undetected and therefore not susceptible to immune attack. Such situations are common to all malignancies[218].

2.15: Gene: HLA-A (Class I)

Gene HLA-DOB.2.2, also known as HLA-DRB3, HLA-DR3B, HLA-DOB.2, HLA-DRB2 or TAP2.2, maps on chromosome 6, at 6p21.3. It encodes an HLA DR-beta-I precursor. The products have Class II MHC antigen activity and are involved in immune response and localize in membrane.

HLA-DRB3 belongs to the HLA Class II beta chain paralogues. This Class II molecule is a heterodimer consisting of an alpha (DRA) and a beta (DRB) chain, both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells. The beta chain is approximately 26-28 kDa and its gene contains 6 exons. Exon one encodes the leader peptide, exons 2 and 3 encode the two extracellular domains, exon 4 encodes the transmembrane domain and exon 5 encodes the cytoplasmic tail. Within the DR molecule the beta chain contain all of the polymorphisms defining the peptide binding specificities.

2.16 Conclusions

In urinary bladder epithelium, both papillary and flat neoplastic lesions contain two cell populations, one superficial and the other invasive, the latter portending an aggressive phenotype with poor prognosis. It is likely that these different cell subsets could (and should) be differentiated by gene expression and gene mutation analysis. This is the subject of greatest diagnostic and prognostic dilemma and also the area in which bladder cancer may prove to be a paradigm for many other epithelial neoplasms, hence yielding insights into general mechanisms of cancer progression that currently eludes study of more complex glandular malignancies.

Presently, immunohistochemical reagents (antibodies, lectins etc.) are widely used in analysing malignancies. In this laboratory, staining of bladder tumour samples is routinely performed using a panel of monoclonal antibodies, including those to the protein products of many of the genes discussed in this chapter. There are numerous examples, particularly in the bladder, of the adjunctive value (both clinical and pathological) of knowing detailed phenotypic features of each individual malignancy. These markers are not only valuable in discriminating normal from reactive and neoplastic, but also in identifying tumours that will progress rapidly from those that are relatively quiescent. For example, a high level of the proliferation marker Ki-67 is seen in metastasising cells, as is the over-expression of Her 2/neu. Decreased levels of pRb indicate that a mutation has occurred and that their tumour supressor gene has been inactivated. The reverse is seen in p53 since increased levels indicate the mutated form because it is more stable than the wild type, resists degradation and accumulates in the nucleus. Loss of expression of CD44, and decreased levels of Class I HLA antigens, are both expected in samples where bladder cancer is present.

Knowledge of gene-structure, particularly from the Human Genome Database, has revolutionized our understanding of genetic factors in many neoplasms including bladder tumours. This knowledge has particularly emphasized the limitations of all antibodies employed to phenotype solid tumours and the necessity to transfer to molecular biologically-based analytical systems as quickly as possible. First, immunohistochemical

systems are labour-intensive and cannot be readily automated. They generate data which are derived from different parts of a tumour and hence are not exactly identical. Second, the antibodies currently employed are frequently poorly-defined with respect to their precise epitopes. Such lack of precision is no longer acceptable. It is important to understand the biological function(s) of the individually spliced domains of many of the proteins involved in the different stages of neoplasia, invasion and metastasis. Third, to continue to employ current immunohistochemical techniques, splice variants that differ between different tumours as well as between a tumour and its normal tissue counterpart require, for their detection, a separate antibody for each distinct spliced variant peptide sequence. Generation of antibodies with necessary levels of specification and sensitivity represent an enormous task and may even be biologically impossible. However, it is the expression and assembly of each spectrum of splice variants that determines the individuality of each tumour.

Finally, during the evolution and progression of an individual cancer, involved genes are frequently both heterogeneous and in flux with respect to their expression. Many genes contain numerous sequences that are not only differentially-spliced but may be under the control of different promoters at different times. Some regions are replicated in many different proteins. Meaningful analysis of such complexity requires the use of techniques significantly different from those currently employed. Those novel techniques will not only provide the necessary specificity to distinguish neoplastic from normal, hence refining the pathological diagnosis but will

also provide the basis for biologically-appropriate therapeutic modulation of bladder cancers - and thereafter of other tissue malignancies.

Chapter 3:

Patients and

Methodology

Transitional cell carcinomas are grouped according to morphological criteria into flat and papillary types. Throughout the Western world, the cause of the relentless rise in transitional cell carcinoma is unknown- particularly since the carcinogenic properties of aniline-based chemicals have been recognized and the substances largely eliminated from industrial and domestic use. A variety of different factors including cigarette smoking have been suggested, as etiological agents, with little robust confirmation. In Liverpool/Merseyside the incidence of bladder cancer is above the national average[219]. The reasons are unknown. However, the high concentration of industries manufacturing and using carcinogenic agents in this geographic region, together with the generally high level of cigarette smoking throughout the UK population, are likely important contributors to the observed pattern of urothelial transitional cell cancers.

For many years, the diagnosis and monitoring of urothelial cell bladder cancer has been made by morphological evaluation; principally the assessment of growth patterns of individual neoplasms according to generally-agreed criteria. However, a major difficulty frustrating subsequent effective clinical management of urothelial cancers is the inability of unassisted morphology to distinguish early lesions that progress rapidly, and hence should receive aggressive systemic or radical local treatment, from those that develop in an indolent manner and can be safely managed more circumspectly. Despite continuous monitoring, transformation of formerly indolent cancers to ones that become invasive cannot be predicted by growth pattern and are only recognised after local or distant dissemination

has occurred. Furthermore, morphological examination cannot distinguish between cancers that will respond to intravesical cytotoxic therapy (mitomycin-C or BCG) and those that are, from the outset, chemotherapy-resistant. These difficulties are compounded even further with respect to the "flat" lesions – either those diagnosed for the first time (and may include non-neoplastic reactive, regenerative or dysplastic lesions) or those that are neoplastic and present following treatment of a previous papillary lesion. Morphologically, these lesions may appear indistinguishable from "normal" bladder mucosa, and yet are known from flow- and image-cytometric studies[220] to contain severe chromosomal and DNA abnormalities with the consequence that neoplasia and invasive malignancy invariably develops in those areas. Even after local or systemic treatment, there are no characteristic morphological features by which the efficacy of a particular treatment might be assessed.

Hence, there is an urgent clinical and pathological need to refine the diagnosis and classification of human bladder cancer in order to provide information that is biologically and clinically relevant to each individual patient and to each cancer than is currently available. Genetic abnormalities are known to correlate with specific forms of bladder cancer. The investigations into molecular biology of bladder cancer will aim to provide an enhanced understanding of the pathogenesis and create an efficient profiling system capable of distinguishing low- and high-risk malignancies within each stage. This attempt to classify patients into increasingly well-defined groups based on characterisation of genetic abnormalities and

anticipated natural history should help individualise treatment strategy with therapy becoming more effective and potentially less toxic.

This approach requires a detailed "phenotypic analysis" in order to identify the particular pathway of carcinogenesis being followed by each cancer and the particular phenotypic stage reached by each cancer at any defined point in time. Therefore, with the objective of breaking this deadlock, a multiparametric phenotypic analysis has been performed using antibodies to a series of marker proteins that are recognized to define aspects of the biological properties of human bladder cancers.

In the United Kingdom, routine use of modern tissue-based techniques to provide a phenotypic profile of individual bladder cancers lags behind the standard practice now followed in more advanced countries such as the USA, The Netherlands, Belgium and some other European countries.

Phenotypic profiling by means of immunohistochemistry (IHC), an antibody-based protein expression assay represents the most important and commonly used 'molecular' technique in diagnostic histopathology[221] and is a powerful approach that provides valuable information on the biological status of individual bladder cancers. IHC is an analytical process relying upon the specificity of an antibody (mono- or polyclonal) directed towards specific antigens present within a given tissue. This process involves incubation of tissue sections with an appropriately diluted primary antibody followed by detection by means of a suitable detection system. Antibody

presence and localisation on the tissue section[222] can then be characterised both for the relative amount and distribution of specific proteins in a particular tissue.

However, IHC remained quiescent as it required fresh or frozen material, until Mason *et al.*[223] reported the ability to detect antigens in formalin fixed paraffin embedded tissues. The widespread diagnostic applicability of IHC was with the discovery of antigen retrieval methods. This process involved proteolytic digestion of tissue sections, followed by microwaving or pressure cooking of tissue sections in a variety of different buffers[224]. IHC is now a powerful investigative tool supplementary to H&E tissue morphology and generates diagnostic accuracy, prognosis and therapeutic information in specific areas where morphology alone remains inadequate[221].

This study examined a panel of potential biomarkers previously reported to provide adjunctive information in several different malignancies which are discussed below:

Ki-67 protein has been demonstrated to be a good indicator of proliferation in a variety of tumours; expressed in all cells in G1, S and G2M phases but not in cells in G0 phase, allowing assessment of the entire proliferating cell pool. Expression of Ki-67, a marker of proliferation, accurately demonstrates the rate of cell proliferation in a lesion. High rates of proliferation are recognized to increase the risk of malignant transformation and progression in a variety of neoplasms and hence regarded as a predictor of tumour "aggressiveness" [225] [226]. Normal bladder epithelium has a low proliferation rate, as defined by Ki-67 expression. Thus, Ki-67 assessment provides a numerical value for cell proliferation that is a quantitative index of progression "risk" for each lesion. The CDKN2a gene (p16) is a critical inhibitor of cyclin CDK complex, and its inactivation is thought to permit progression through G1/S cell-cycle checkpoints[227] [228]. This loss of p16 function leads to the loss of Rb tumour suppressor function and, consequently up-regulation of cell cycling[227] [229]. It is also at the G_1/S restriction point that wild-type p53 (in response to cell damage) induces WAF1 gene transcription with the resulting $p21^{waf1}$ protein binding and inactivating the cyclin/cdk complexes. The consequent accumulation of hypophosphorylated pRb blocks E2F-dependant transcription leading to cell cycle arrest[230]. Use of immunohistochemical markers to tumour suppressor genes p53, Rb and PTEN allows accurate identification of genotypically

abnormal lesions- and hence of differentiating between reactive and potentially neoplastic epithelium. Expression of p53 is a particularly powerful discriminator in early lesions, being absent in reactive epithelium and positive in approximately 80% of neoplasms, which cannot otherwise be segregated according to unassisted morphological criteria [231 232]. PTEN is a tumour suppressor gene mapped to chromosome 10q. Studies for loss of heterozygosity or allelic imbalance have shown deletions in ⅓ of muscle invasive tumours but only in <7% of superficial tumours[233 234].

Bcl-2[20] and cell-surface receptor c-erb-B2[235] appear to exhibit a reciprocal relationship in neoplastic urothelial lesions. Loss of normal expression of bcl-2 by urothelial basal epithelial cells and de-novo expression of c-erb-B2 are early cellular changes that accurately define the neoplastic phenotype and hence predict development of bladder cancer-of either flat or papillary types. The apoptotic modulator Bcl-2 proto-oncogene appears to increase cell longevity by regulating a downstream event leading to the induction of apoptosis, thereby allowing cells to escape apoptosis[208]. The bcl-X gene is a homologue of the bcl-2 gene which has demonstrated different patterns of expression and thus suggested to regulate different stages in the execution-phase of cellular death[199]. Bcl-X_L is the longer alternative protein transcript encoded by bcl-X and suppresses cell death with the shorter bcl-X_S which promotes apoptosis[208].

Expression of homeostatic regulator protein hsp-27 [236] is an accurate and powerful marker of bladder urothelial cells, as well as being intimately

involved in modulating the response of cells, of both normal and neoplastic types, to expression and activation of oestrogen receptor proteins. Potentially, it is an extremely valuable reagent with which to identify single-cell invasion across the basement membrane and into the sub-epithelial stroma; thus changing the pathological grade of an early cancer from that of pTa (in-situ cancer) to pT1 (microscopically superficially-invasive). These are notoriously difficult lesions to diagnose accurately such that use of an independent and objective marker of epithelial cell invasion is highly desirable and would be extremely valuable.

Finally, the role of oestrogen receptor expression in urothelial carcinoma has not been extensively analyzed. Oestrogen function is mediated through two specific intracellular receptors ER- α and ER- β which play important roles in mediating nongenotropic oestrogenic effects[237]. Studies[238 239] have shown higher levels of ER expression in human urothelial carcinoma suggesting that oestrogen receptors may play a more important role in the biology of human bladder cancer.

Therefore, the objective of this part of the overall study was to test the hypothesis that objective phenotypic analysis of urothelial lesions with selected marker proteins, using commercially-available antibodies, identifies some of the important proteins responsible for promoting and determining the stage of individual human bladder cancers. It was anticipated that the data from the selected panel of antibodies would provide novel reliable information, not otherwise available, to accurately predict the

pathological and clinical behavior of particular urothelial neoplasms and hence improve the management strategies of individual patients with bladder cancer.

3.2: Patients

3.2.1: Cases Studied

A cohort of 138 patients referred for management of urothelial neoplasia to the Multidisciplinary Team Meeting (MDT) for Urological Malignancies at the Royal Liverpool and Broadgreen University Hospitals (NHS) Trust, Liverpool, UK, were recruited sequentially and unselected to this study. These were patients who had had bladder tumour diagnosed following investigations for visible or non-visible haematuria, other lower urinary tract symptoms such as urinary frequency or recurrent urinary tract infections. All patients had an initial cystoscopy evaluation of the bladder under general anesthesia with resection of bladder tumour for conventional histological reporting. These patients were reviewed longitudinally over time with respect to their clinical and pathological status at each presentation to the MDT following the initial histopathological diagnosis of urothelial carcinoma. All original H&E stained tissue sections, and their accompanying paraffin wax tissue blocks, were retrieved from the archives of the Department of Pathology at the Royal Liverpool and Broadgreen University Hospitals (NHS) Trust. The morphological appearances of each biopsy specimen were reviewed to confirm the diagnosis and to ensure consistency of the reported pathological grade and stage. These tissue blocks were held within the Pathology archive in the Department of Pathology, University of Liverpool. Histological sections were cut at 4 μm thickness from formalin-fixed and paraffin wax-embedded tissues and

processed as described previously[223]. Additional sections were then cut from the paraffin wax-embedded tissue blocks and stained using the panel of selected antibodies.

All 368 tissue sections were examined independently by urological pathologists for histological staging using the H&E stained specimen. The biomarker grading was undertaken independently with positive staining scored from 1-3 (0 being negative) with three being strongly positive.

Diagnosis		Number of patients
Normal (Control)		17
No abnormality		11
Chronic inflammation		5 (1)*
Non-muscle Invasive Carcinoma	Dysplasia	4
	CIS	9 (1)*
	pTa	38 (1)*
	pT1	27 (4)*
Muscle invasive Carcinoma	pT2	16
	≥pT3	4
TOTAL		**131**

(n)*- non-muscle invasive carcinoma to invasive disease;

Table 3.1: Patient Demographics- FINAL COHORT

3.2.2: Controls

Control bladder tissue specimen were selected randomly but over the same period of time as the cases studied, from patients biopsied for histologically-confirmed non-neoplastic conditions of the urinary bladder. These specimens were assessed morphologically and by clinical follow-up to be free from neoplastic disease, particularly of the urinary bladder. Since all those patients presented for investigation of probable underlying inflammatory disease, but not neoplasia, they were typically both female and also younger than the cases studied.

For this analysis, it was irrelevant that these control cases are not age- and gender- matched to the cases studied, since no therapeutic outcome was being assessed. Therefore, these controls were appropriate to exclude any effects of neoplastic disease. The purpose of this study was to identify phenotypic changes that could be attributed to neoplastic disease of the urinary bladder irrespective of age, gender or the presence of inflammation.

3.3: Ethical Approval

This study was approved by the Liverpool (Adult) local Research Ethics Committee (LREC) in their advisory capacity to the Cheshire & Merseyside Authority (03/04/016/A)

3.4: Immunohistochemical Staining

Sections of each specimen were cut at 4μm from formalin-fixed, paraffin-embedded tissue blocks onto APES (amino-propyl tri-ethoxysilane) –coated glass slides and dried overnight in an oven at 56^0C. The fixation and processing of tissues can have a 'masking' effect upon some of the antigens within them. Various pre-treatments have been devised which may be applied to tissue sections to reverse this masking effect, and allow primary antibodies to recognize and bind to their target antigens.

3.4.1: Pre-treatment:

Sections were treated with methanol/H_2O_2 for 12 minutes to remove the paraffin wax followed by rehydration through graded ethanols. Endogenous peroxidase activity was blocked by immersion in a 3% (w/v) solution of H_20_2 in methanol for 12 minutes. Sections were then rinsed in tap water followed by deionised water.

Pre-treatment with trypsin and calcium chloride (Becton-Dickinson, Difco 0152-13), pepsin (Sigma P-7000) and protease XXIV (Sigma P-8038) was undertaken for 15-30 minutes at 37^0C.

3.4.2: High Temperature Antigen Retrieval

High temperature antigen retrieval was performed using a domestic stainless-steel pressure cooker at full-pressure for 3 minutes in 10 mM EDTA (Ethylenediaminetetraacetic acid) solution (pH 7.0) and immediately cooled by running the pressure cooker into a sink of cold running tap water.

High Temperature Antigen Retrieval (HTAR) combined with Trypsin Digestion:

Sections become extremely sensitive to the effects of proteolytic digestion after they have been subjected to HTAR. This is especially true of sections subjected to HTAR using the pressure-cooker in which digestion time must be controlled very precisely. It is preferable to use the microwave method when HTAR is to be combined with digestion[240].

HTAR was performed as described above. The sections were then washed in tap water and rinsed in deionised water. Sections were then placed in trypsin solution at 37^0C for an exactly timed 20 minutes and then washed well in running tap water.

3.4.3: Immunohistochemistry

The technique used in our laboratory was the commercially available DAKO EnVision™+ System, Peroxidase (DAKO EnVision™+ System, HRP) for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissue preparations.

The DAKO EnVision™+ System, HRP is a two-step immunohistochemical staining technique (Figure 3.1).

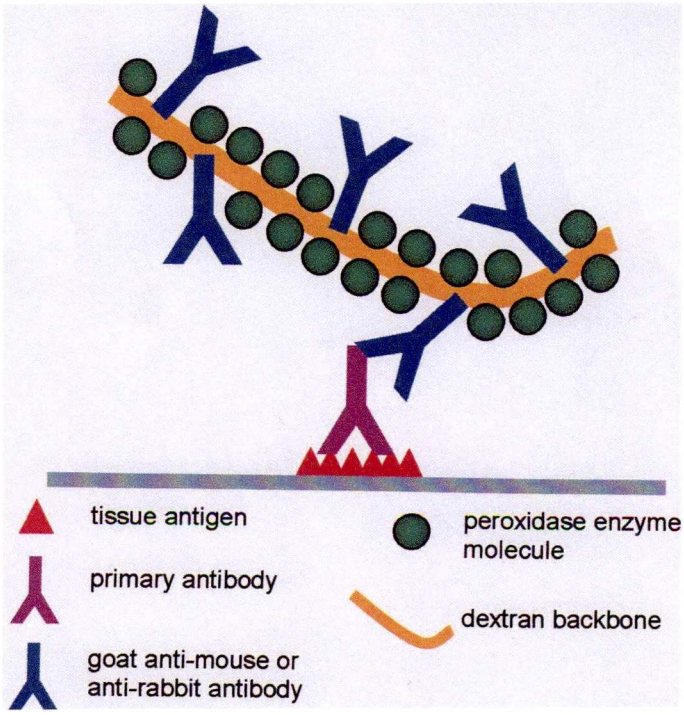


Figure 3.1: DakEnVision™ + System, HRP two-step immunocytochemical staining technique

This system is based on a horse-radish peroxidase-labelled polymer that is conjugated to secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequently, nonspecific staining resulting from endogenous avidin-biotin activity is eliminated or significantly reduced. The kits were species specific; EnVision (mouse) used with mouse monoclonal antibodies and EnVision (rabbit) with rabbit polyclonal primary antibodies.

After appropriate pre-treatment and HTAR as described above, the slides were transferred to an automatic immunostainer (Sequenza®). After equilibration in fresh Tris Buffered Saline comprising 0.05M Tris (pH 7.6) containing 0.12M NaCl and 0.05% Tween-20 (TBS-T), sections were incubated with primary mouse or rabbit antibody or negative control reagent (100μL of 5% bovine serum albumen was applied), followed by incubation with 100μL of the labelled polymer, using two sequential 30-minute incubations. Staining was completed by a 10 minute incubation with 100μL of 3,3'-diaminobenzidine + substrate-chromogen and washed in deionised water and running tap water. Finally, the sections were counter stained with haematoxylin solution. The end results were a brown-coloured precipitate at the sites of antigen-antibody binding and the nuclei staining blue.

3.4.4: Primary Antibodies

These were selected based on the current evidence in literature and commercial availability. Staining protocol for the cell-cycle regulatory proteins were all monoclonal mouse anti-human antibodies. Table 3.2 lists

the primary antibodies used for staining the various proteins. Samples with known positive reactivity for each monoclonal antibody were used as positive controls. As a negative control, a section was processed in which the primary antibody was changed by PBS.

Protein	**Primary Antibody**
Ki-67	MIB-1; 1:200, DAKO, Carpinteria, CA[241]
ER- α	Mouse monoclonal 1:200, Bio- Genex, San Ramon, CA[241]
ER- β	monoclonal antibody ER(Dako, K1900)[238]
Hsp-27	Mouse monoclonal p27kip1 (Dako, Glostrup, Denmark)[242]
p53	Mouse monoclonal PAb 1801, 1:50 (Dianova, Hamburg, Germany)[138]
pRb	RB1, Dako, 1:75[243]
p21	Ab1, Oncogene Science, Cambridge, MA, 1:20[244]
p16	Mouse monoclonal, NeoMarkers, 1:50[245]
PTEN	Polyclonal anti-PTEN, 1:100 (Upstate Technologies)[246]
c-erb-B2	Polyclonal, 1:200 (Dako Corp., Carpinteria, CA)[247]
Bcl-2	Mouse monoclonal, 1:40, Dako Laboratories, Carpentaria, CA[199]
Bcl-x_L	Polyclonal, 1:150, Zymed Laboratories, San Francisco, CA[248]

Table 3.2: Panel of Biomarkers

3.5: Qualitative Scoring of Markers

The biomarkers were independently assessed for each tissue section as either negative, weakly positive or only focally positive (low-level expression), or strongly positive (high-level expression) and scored as 0, 1, 2 or 3, respectively with 1- being mildly positive and 3- being strongly positive. Specimens were considered positive only when at least 5%-10% of the contained urothelial cells (either normal or malignant) unequivocally expressed the biomarker.

3.6: DATA ANALYSIS

Individual cancer diagnosis and biomarker expression were used in the statistical analysis of the collated data.

The cancer patients were categorised into 5 distinct groups based on the grade of malignant transformation and stage of tumour invasion. Patients who were known to have a history of malignancy and a negative index biopsy which had been undertaken for suspected recurrent malignancy (no abnormality detected) or histological evidence suggestive of varying degree of chronic inflammation were grouped together. Dysplasia, carcinoma-in-situ and pTa bladder tumours were categorised together, pT1 bladder tumours considered a separate entity; and muscle invasive tumours, a fifth group.

This grouping of the patients was aimed at enhancing the relationship between the stage of the disease (1 representing the normal controls, 2 being the group with no detected abnormality or chronic inflammation through to 5 representing muscle invasive disease) and the biomarkers with an emphasis on the non-muscle invasive urothelial cancer (pTa/ pT1 tumour). The biomarker scoring system was dichotomised with zero and one classed as negative and two and three considered positive.

Calculations were performed using the Statistical Package for the Social Science Version 17.0 (SPSS Inc., Chicago, IL, USA). Non-parametric test statistics were used to identify the significant association between the urothelial carcinoma stages and the corresponding biomarkers. The resulting stage groups were used as dependent variables in the binary calculations and the calculations were repeated for different methods to ensure numerical accuracy and reliability.

Chapter 4:

Results

This study cohort comprised of 368 index bladder specimens from 138 patients assessed pathologically for urothelial carcinoma. However, the tissue blocks from six patients contained too little material and hence were inadequate for immunohistochemical staining and analysis. These were therefore excluded from the final analyses. One patient diagnosed to have squamous cell carcinoma was also excluded from the study.

The final cohort comprised 131 patients with adequate biopsy tissue for the complete analysis with the panel of 12 biomarkers. 114 patients had diagnosed urothelial carcinoma and 17 patients who were considered as controls. These latter were patients with benign disease who had been investigated for inflammatory/infective disorders.

The control group comprised 19 index bladder biopsies from 17 patients. The mean age of this group was 50 years (range 21-86 years; SD 20). 12 were female with only 5 being male. In the cancer group, were 312 index biopsies/resections deemed suitable for analysis. The mean age of the cancer group was 68 years (range 30-91years; SD 13). 82 of these patients were male and 32 were female.

Within this group, 79 patients were diagnosed pathologically to have non-muscle invasive urothelial carcinoma. Of these, seven developed invasive disease. On primary bladder tumour resection, 20 patients were diagnosed to have muscle invasive disease and later underwent radical treatment. A further two patients were treated conservatively with static disease.

In the non-muscle invasive disease group over 13 years, there was an increase in stage (e.g: pTa-pT_1) in seven patients. No change in the stage was recorded in eight patients and a decrease occurred in stage in six. 15 patients had either a benign or inflammatory histology when urinary bladder lesions were biopsied/resected as part of their urinary bladder follow-up.

The immunohistochemical staining characteristics are contained in Table 4.1. The test data (Kruskal-Wallis Test) comparing the individual biomarkers and the stages are contained in Table 4.2. Of the total panel of biomarkers, the indices Ki-67, p53, bcl-2, Bcl-X_L, ER-β and c-erb-B2 indices revealed significance ($p < 0.05$) with respect to prognosis suggesting that these biomarkers may have independent association with tumour stage. On decreasing the p-value to $p \leq 0.001$, only Ki-67, p53, bcl-2 and c-erb-B2 were identified to have a distinct ordinal relationship between the marker values and the tumour stage.

	Ki-67	p 53	Rb	Bcl-2	p21	p16	Bcl-X_L	PTEN	Er α	Er β	c-erb-B2	Hsp-27
Neg (0)	88	44	29	179	105	37	104	96	128	161	23	8
1	91	76	60	70	60	19	10	63	2	66	44	19
2	80	86	14	28	37	9	5	54	0	37	82	51
3	31	95	197	24	18	10	2	67	0	25	135	179
Total	290	301	300	301	220	75	121	280	130	289	284	257

Table 4.1: Expression of Individual Biomakers in the Study Cohort

	Ki-67	p 53	Rb	Bcl-2	p21	p16	Bcl-X_L	PTEN	Er α	Er β	c-erb-B2	Hsp-27
Chi-Square	64.9	16.35	5.45	53.8	2.7	7.03	10.8	7.0	0.0	10.6	24.2	2.5
Df	4	4	4	4	4	4	4	4	4	4	4	4
p value	.00	.00	.24	.00	.62	.14	.03	.13	1.0	.03	.00	.65

Table 4.2: Individual Biomarkers and Analysis with Stage Group

(Kruskal-Wallis Test)

4.2: Control group

The control group consisted of 17 patients who had had a total of 19 bladder biopsies. The biomarker staining characteristics of this group of patients are in Table 4.3. 15 patients had only one bladder biopsy with no secondary or follow-up procedure performed due to the benign histology. Two patients had two biopsies which were taken during the same procedure.

To identify their significance, all immunohistochemical staining was dichotomised as negative (scores of zero and one) and positive (two and three) and subsequent analysis performed. Descriptive statistics suggest that the biomarker expression for true normal would describe the following pattern:

POSITIVE-- Rb, erb-B2, Hsp-27

NEGATIVE—Ki-67, p16, Bcl-X_L, ER-α

	Ki-67	**p 53**	**Rb**	**Bcl-2**	**p21**	**p16**	**Bcl-X_L**	**PTEN**	**Er α**	**Er β**	**c-erb-B2**	**Hsp-27**
Neg(0, 1)	19	11	3	6	16	14	2	14	2	11	2	1
Pos (2, 3)	0	7	16	11	3	1	0	4	0	8	17	18
%post	0	39	84	65	16	7	0	22	0	42	90	95

Table 4.3: Expression of Indivudal Biomarkers in normal (Control) patients (17 patients, 19 biopsies)

4.3: Non-Muscle invasive urothelial carcinoma group

This group includes the morphological system of neoplasia and involving at most the lamina propria. The analysis was performed only after combining various stages into groups. Thus dysplasia, carcinoma in-situ and pTa disease were classified into one group. pT_1 disease was considered as a separate group.

Four patients diagnosed with dysplasia, one of who later developed pT_1 disease seven months later. Nine had carcinoma-in-situ (CIS) disease with all but one having static disease. One patient with CIS developed muscle invasive disease at four years from initial diagnosis. The majority of patients with superficial disease had pTa disease (38 patients). Four of these patients developed pT_1 disease (mean 6.5years, range: 2-11years) and one patient developing pT_2 disease (after 13years).

27 patients were diagnosed to have pT_1 disease. Four of these patients developed muscle invasive (pT_2) disease. Three were upstaged on the immediate re-resection (<6 months-<1year) and the fourth patient was upstaged within three years. In this sub-group the confounding factor is that these patients could have been under-staged with the initial resection biopsy as the interval between the initial biopsy and the second/further biopsies resulting in the upstaging was a short duration. pT_1 disease was down-graded in three patients to chronic inflammation, dysplasia and pTa disease.

Descriptive statistics suggest that the biomarker expression for the non-muscle invasive urothelial carcinoma (Dysplasia, CIS and pTa disease) would describe the following pattern (Table 4.4):

POSITIVE—p53, Rb, c-erb-B2 and hsp-27.

NEGATIVE—Bcl-2 and Bcl-X_L.

In the pT_1 disease subset, changes in the biomarker expression pattern were more pronounced (Table 4.4):

POSITIVE—p53, Rb, c-erb-B2 and hsp-27.

NEGATIVE—Bcl-2 and Bcl-X_L.

There were more positive cases of Ki-67, p53 and Rb in comparison to the dysplasia, CIS and pTa disease group.

Statistical analysis of the biomarker expression over three stages (Table 4.5) using a 2-tailed Mann-Whitney U test model compared normal group (control) histology versus group 3 (dysplasia, CIS & pTa) and then versus pT_1 disease. Initial comparison of the normal (control) group versus group 3 (dysplasia, CIS and pTa disease) showed that of the total panel of biomarkers in early non-muscle invasive disease; Ki-67, Bcl-2, p16, PTEN and ER-β revealed significance ($p<0.05$) with respect to prognosis suggesting that these biomarkers may have significant association with tumour stage. On decreasing the p value to ≤ 0.001; only Ki-67 and Bcl-2 were identified to have a distinct ordinal relationship between marker values and the tumour stage. In comparing the controls versus pT_1 disease; Ki-67, p53, Bcl-2 and p16 were significantly ($p<0.05$) correlated. However, only

Ki-67 and Bcl-2 maintained this association when the p-value was decreased to $p \leq 0.001$.

The analysis also revealed a significant association with Ki-67 and p53 when comparing dysplasia, CIS and pT_1 disease with pT_1 urothelial carcinoma.

A significant increase was identified in Ki-67 expression from non-neoplastic tissue through early non-muscle invasive carcinoma to pT_1 disease. Up-regulation of p53 and p16 was also seen. There was loss of bcl-2 expression and ER-β down-regulation. ER-α expression was negative across all stages.

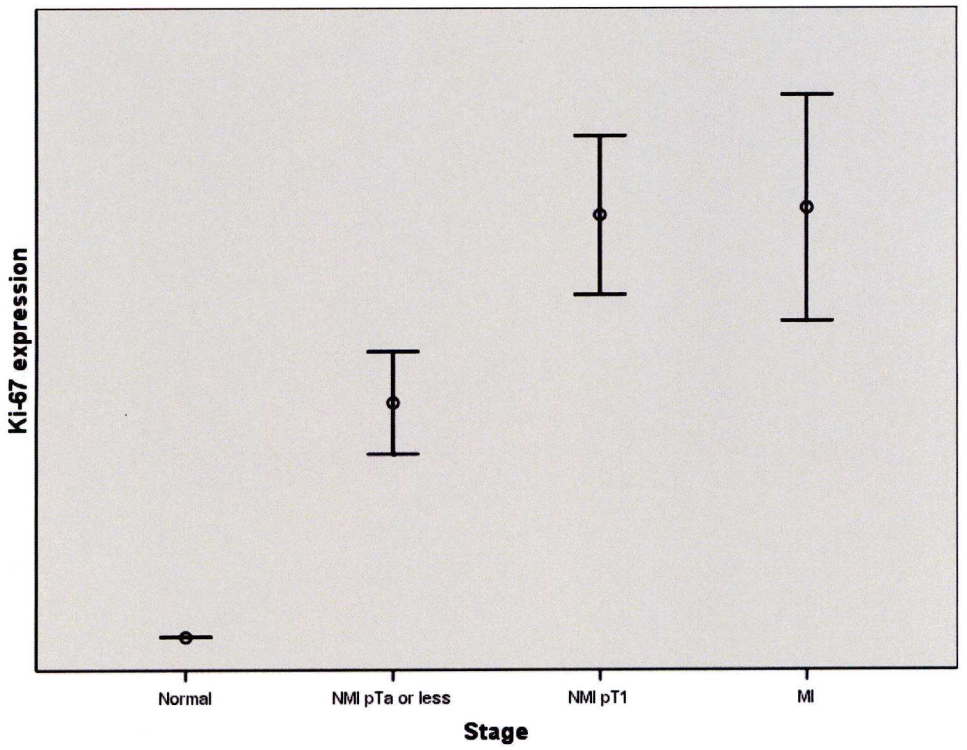


Figure 4.1: Ki-67 expression pattern by stage

(NMI- Non-muscle invasive; MI-muscle invasive disease)

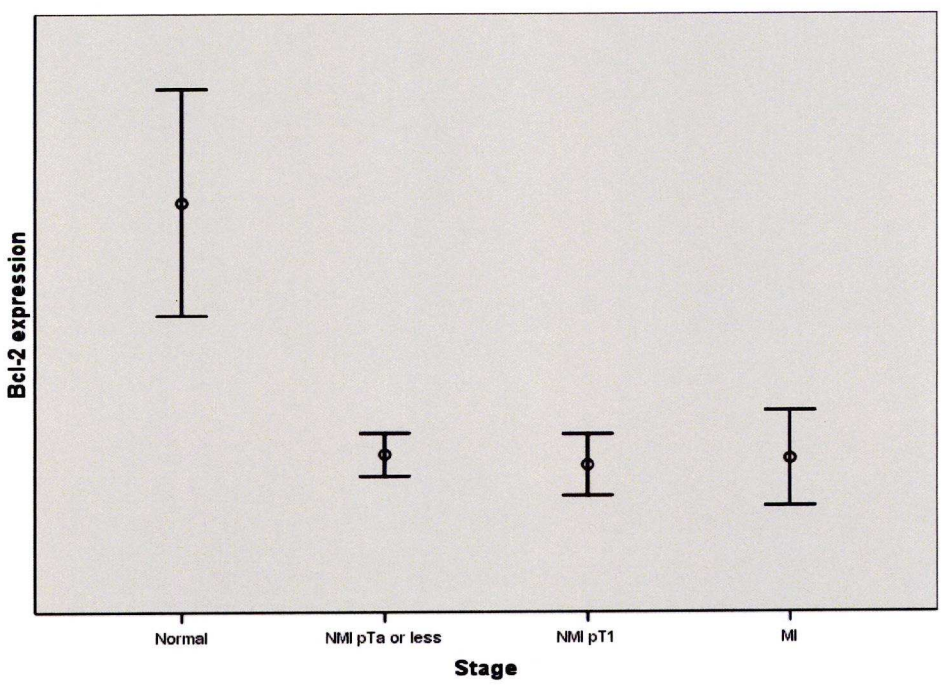


Figure 4.2: Bcl-2 expression pattern by stage

(NMI- Non-muscle invasive; MI-muscle invasive disease)

	Ki-67	p 53	Rb	Bcl-2	p21	p16	Bcl-X_L	PTEN	Er α	Er β	c-erb-B2	Hsp-27
Dysplasia/ CIS/ pTa	50 (39)	80 (62)	84 (64)	11 (8)	23 (26)	10 (37)	1 (2)	56 (46)	0 (0)	26 (21)	102 (84)	94 (90)
pT_1	34 (71)	40 (78)	39 (77)	3 (6)	11 (31)	5 (39)	2 (10)	17 (36)	0 (0)	10 (20)	44 (86)	41 (87)

number (%)

Table 4.4: Positive expression of Individual Biomarkers in non-muscle invasive urothelial carcinoma (79 patients, 185 biopsies)

p values	Ki-67	p 53	Rb	Bcl-2	p21	p16	Bcl-X_L	PTEN	Er β	c-erb-B2	Hsp-27
N□ vs Gp 3*	0.00	0.07	0.08	0.00	0.33	0.03	0.87	0.06	0.05	0.51	0.49
N vs pT_1	0.00	0.00	0.49	0.00	0.24	0.05	0.66	0.29	0.06	0.72	0.37
Gp 3* vs pT_1	0.00	0.03	0.11	0.60	0.64	0.93	0.10	0.27	0.87	0.66	0.68

*Group 3-(Dysplasia, CIS, pTa). N□-normal

Table 4.5: Comparison between Non-Muscle Invasive Urothelial Carcinoma Groups and Normal Bladder biopsies

4.4: Muscle invasive urothelial carcinoma group

This group comprised of patients diagnosed to have pT_2, pT_3 and pT_4 urothelial carcinoma at initial diagnosis. Within this group, 20 patients were diagnosed with muscle invasive disease. 16 had pT_2 disease with three having pT_3 and one with pT_4 urothelial carcinoma. Almost all these patients were then treated radically with either cystectomy or radiotherapy. Two with pT_2 disease were treated conservatively and remained with stable disease at ive and eight years follow-up. Seven patients initially diagnosed with non-muscle invasive bladder carcinoma and then developed muscle invasive disease were included in this group for analysis.

In these patients with aggressive muscle-invasive urothelial carcinoma; the biomarker profiling pattern was as below (Table 4.6):

POSITIVE— Rb, Bcl-2 and hsp-27.

NEGATIVE— ERβ.

	Ki-67	p53	Rb	Bcl-2	p21	p16	Bcl-X_L	PTEN	Er α	Er β	c-erb-B2	Hsp-27
Negative (0, 1)	7	9	7	2	13	4	9	17	12	27	10	4
Positive (2, 3)	18	19	21	25	6	1	3	10	0	1	16	19
% positive biopsies	72	70	75	93	32	20	25	37	0	4	62	83

Table 4.6: Expression of Individual Biomarkers in Muscle Invasive Urothelial Carcinoma

Mann-Whitney U test analysis (Table 4.7) suggests a significant up-regulation of Ki-67 and Bcl-2 with marked loss of ER-β and c-erb-B2 in muscle invasive urothelial carcinoma in comparison with non-neoplastic bladder biopsies. This strong association persisted even with $p \leq 0.001$ except for c-erb-B2 ($p<0.05$). In comparison with non-muscle invasive (dysplasia to pT_1 disease) urothelial carcinoma, the urothelium significantly gained Ki-67 and Bcl--X_L with down regulation of ER-β and c-erb-B2 with the urothelial carcinoma becoming muscle invasive.

Seven patients with non-muscle invasive carcinoma developed muscle invasive disease. Sub-group analysis revealed a significant association with prognosis with the informative biomarkers being Rb, p21, PTEN and hsp-27. Increased expression of Rb, p21 and PTEN with down regulation of hsp-27 suggests an increased likelihood of developing muscle invasive disease.

p values	Ki-67	p 53	Rb	Bcl-2	p21	p16	Bcl-X_L	PTEN	Er β	c-erb-B2	Hsp-27
Normal vs MI*	0.00	0.06	0.45	0.00	0.26	0.40	0.44	0.30	0.00	0.04	0.23
pT1 vs MI*	0.00	0.53	0.27	0.87	0.65	0.47	0.00	0.42	0.03	0.01	0.35
NMI□ vs MI*	0.03	0.87	0.432	0.96	0.72	0.45	0.01	0.57	0.03	0.01	0.40

□non-muscle invasive carcinoma; *muscle invasive carcinoma

Table 4.7: Comparison between muscle invasive urothelial carcinoma and non-muscle invasive disease groups and normal bladder biopsies

4.5: Chronic inflammation / No abnormality detected ("Normal")

There were 77 bladder biopsies where the histological diagnosis was chronic inflammation or "normal". These were diagnosed in patients with strong suspicion of urothelial malignancy or a previous treated urothelial malignancy. The possibility of abnormal biomarker profiling in these biopsies where there is a significant deficiency in routine H&E staining failing to detect/ differentiate between chronic inflammation, dysplasia and CIS precluded the inclusion of this category in the final analysis. Statistical analysis did reveal that this group were a distinct entity from both the 'true' normal biopsies (Bcl-2, PTEN and c-erb-B2-- $p<0.05$) as well as the early (dysplasia, CIS and pTa) non-muscle invasive disease (Ki-67, p53, bcl-2 and c-erb-B2—$p<0.05$).

Chapter 5:

Urinary Bladder Cancer Progression

Predicted at Diagnosis by Quantitative Analysis of

Ki-67 Protein Expression

UK rates for urinary bladder cancer have fallen since their peak in the 1990's[105]. Although the incidence of TCC remains biased towards men, the prevalence of this disease in the U.K. has altered to a male:female gender ratio that is presently 5:2. There is concern that altered environmental factors promote development and progression of TCC through hitherto unrecognized mechanisms, especially in women [249 250]. Formerly regarded as a disease of the elderly, TCC is diagnosed more frequently in younger persons, increasing its socio-economic impact. Although the five-year survival for superficial tumors (pT_a-pT_{1a}) is good at 80-90%, survival declines to <50% with muscle invasion ($\geq pT_2$). For bladder-confined tumors, treatment is limited to intravesical BCG [251], mitomycin C, a combination of both [252] or to radical cystectomy [253]. Radical radiotherapy for pT1-G3-NX-M0 disease is no more effective than conventional conservative treatments [254]. Furthermore, current therapies are complicated by severe local pain, give rise to systemic complications [255 256] and their long-term benefits are limited [256]. Thus, there is a need to target therapies to those patients predicted to have progressive disease. While histological assessment at diagnosis is helpful to clinicians, it is not a good prognostic predictor of disease progression or outcome [257] because morphologically similar bladder cancers are phenotypically distinct and behave differently.

To assess tumor proliferation, immunohistochemical studies have been used as a surrogate to predict tumor aggressiveness and prognosis of other malignancies [258 259]. In addition to bladder, [260 261], the Ki-67 protein, detected

by monoclonal antibody MIB-1, has been demonstrated to be a good indicator of proliferation in a variety of tumors including bladder neoplasia [262 263]. Ki-67 is expressed in all cells in G_1-S and G_2M phases but not in cells in G_0 phase, allowing assessment of the entire proliferating cell pool [264]. The Ki-67 index (i.e. percentage of a population of cells expressing nuclear Ki-67) has been used in prostate cancer to evaluate likely clinical behavior and prognosis [258 265]. In transitional carcinoma cells, Ki-67 exhibits an inverse relationship with expression of cell-cycle checkpoint gene CCND1 [261]. The 'point counting' approach commonly employed to evaluate Ki-67 expression is both time-consuming and raises the probability of sampling error. Other indices have been employed to assess proliferation rates, with varying validity [265-267] so that reproducibility has been difficult to assess.

To generate objective numerical data, we developed an interactive computer-based analysis technique to quantify Ki-67 expression in a reproducible manner. We assessed Ki-67 expression relative to grade and stage and have analysed variations in quantifying Ki-67 when comparing the detailed numerical data with semi-quantitative impressions derived following subjective assessment of staining. Thus, the aim of the present study was to test the hypothesis that, in superficial bladder cancer, assessment of the region of maximum cell proliferation by Ki-67 expression in a diagnostic biopsy specimen is a reliable predictor of cancer progression and hence could be used routinely as one of the parameters to determine individual bladder cancer management.

5.1: Patients and Methods

This study analysed a sub-cohort of 86 of the total 368 bladder biopsy specimens derived from 35 of the 138 patients with long-term follow-up received in the Department of Pathology at The Royal Liverpool University Hospital, Liverpool, UK. These were Cases were entered into the data-set on their first schedules appointment, either as a new referral or on routine follow-up. For patients with the prior diagnosis of bladder cancer, details of their previous pathological investigations were retrieved, reviewed and entered into the database. Thus, a retrospective and prospective cohort of patients with histologically-confirmed TCC under active surveillance was compiled together with a series of patients in which malignancy was excluded histologically. Paraffin-wax blocks of morphologically non-neoplastic archival bladder tissues and corresponding urothelial cancers were routinely stained with haematoxylin and eosin (H&E) to establish the histopathological diagnosis. All cancers were classified according to the TNM classification [268]. Following referral, all patients were managed in a consistent manner through weekly multidisciplinary team (MDT) meetings according to conventional protocols.

5.2: Immunohistochemistry

Tissue sections serial to those stained with H&E and used for initial diagnostic purposes were stained for Ki-67 expression [258]. Tissue sections

were incubated for 60 minutes at room temperature with biotinylated monoclonal antibody MIB-1 (Dako Ltd, Denmark) diluted 1:50 in phosphate-buffered saline (PBS) before immersion in a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Fluka AG, Switzerland) at 250 μg/ml in 0.04% (v/v) H_2O_2 in methanol for 5 minutes to develop the chromagen. Negative controls comprised sections of benign and malignant urothelium processed identically with the exception that the MIB-1 antibody was substituted with PBS.

5.3: Determination of baseline scores

Following diagnosis, all H&E-stained tissue sections were reviewed by a senior pathologist (CSF) to confirm that adequate tumor was present on each section and that the diagnosis, grade and stage were standardised and correct. All stained tissue sections were then analysed, the slides being assigned independent scores, the observer being blinded to the data accruing within the programme. The baseline score was that subjectively assigned by the pathologist on a scale of 1-4 with "1" being no expression and 4 being highly proliferative. A 10% cut-off was used to distinguish cases considered negative from those with positive scores (i.e. at least 10% of nuclei had to stain to be awarded a score >1). Following collection, these Ki-67 scores were compared with pathological stage and grade.

5.4: Determination of proliferation indices

All tissue sections initially scored by myself and Professor Foster were analysed using a Zeiss KS300 image quantification system (Carl Zeiss Ltd, Welwyn Garden City, UK) at x40 magnification. A computer programme developed within the Division of Pathology allowed semi-automated assessment of positively stained Ki-67 nuclei. For all cases examined, the density of nuclear staining for Ki-67 was adjusted against a standard to ensure consistency, particularly in accepting the low cut-off intensity. Thereafter, two new indices were developed to enable automated characterisation of proliferation: Ki-67 percent (Ki-67p) being the ratio of stained to unstained nuclei in the area of a selected field. Each area was individually defined by the investigator to include only areas of epithelium (either benign or malignant) and to exclude all non-epithelial tissues. Conversely, Ki-67 density (Ki-67d) was recorded as the ratio of "positive" nuclei within the same total area occupied by the malignant epithelial cells.

A total of 102 tissue sections from 56 patients were scored. Five separate areas in each tissue section containing adequate malignant or benign urothelium were analyzed. Sections that did not contain five distinctly separate areas of urothelium for analysis were excluded from the study. As a consequence, 86 sections containing adequate urothelium for five separate and consecutive readings and were included in this study. The mean values of the five readings were used for analysis.

Conventionally, during statistical analysis, all readings (for both Ki67p and Ki67d) were assigned to one of four groups: (0-0.01 = 1, 0.01-1 = 2, 1-2 = 3 and >2 = 4) where Group 1 was negative and Group 4 was strongly positive. These groups were given equal weighting and selected to represent the values assigned by the pathologist during histological assessment of each field. Absence of staining was not assigned the score "0" but "1" because of the mathematical implications involved in handling the term "zero".

5.5: Statistics

Analysis of the data on the stage and grade at diagnosis, together with indices and baseline scores was performed using the Statistical Package for the Social Science Version 14.0 (SPSS Inc., Chicago, IL, USA). Correlation studies were undertaken with Spearman's test (r_s). The Kruskall Wallis test was used for group comparison. For all comparisons $p < 0.05$ was considered statistically significant.

5.6: Results

Tissues for analysis were available from 35 patients with urinary bladder cancer. Of all the tissue sections stained and examined, 86 slides were included in the analysis, all others being excluded wherever epithelial tissues were insufficient to fulfil the requirement of at least 5 distinct fields for analysis. Of these, 14 sections were histologically normal, 9 inflammatory, 8 dysplastic, 39 CIS/superficial bladder cancer and 16 invasive cancers. Mean age was 76 years (range: 51-94 years). There was no significant difference in age between patients with differing grade or stage ($p=0.34$). Positive nuclear staining (Score >1) was observed in 75 of the 86 sections in at least one of three parameters. 11 slides gave zero readings (Figure 5.1) in all three parameters with non-malignant disease. However, dysplastic lesions were frequently accompanied by a focal increase in proliferation (Figure 5.2).

A breakdown of the major groups is given in Table 5.1. Ki-67p ($p<0.01$) and Ki-67d ($p<0.01$) both showed a significant correlation with tumor grade and stage. Morphologically identical lesions, both papillary and flat exhibited a wide range of proliferative activity that could not be predicted from morphological features, whether nuclear or cytological (Figures 5.3 and 5.4). This was true also for the baseline scores ($p<0.01$).

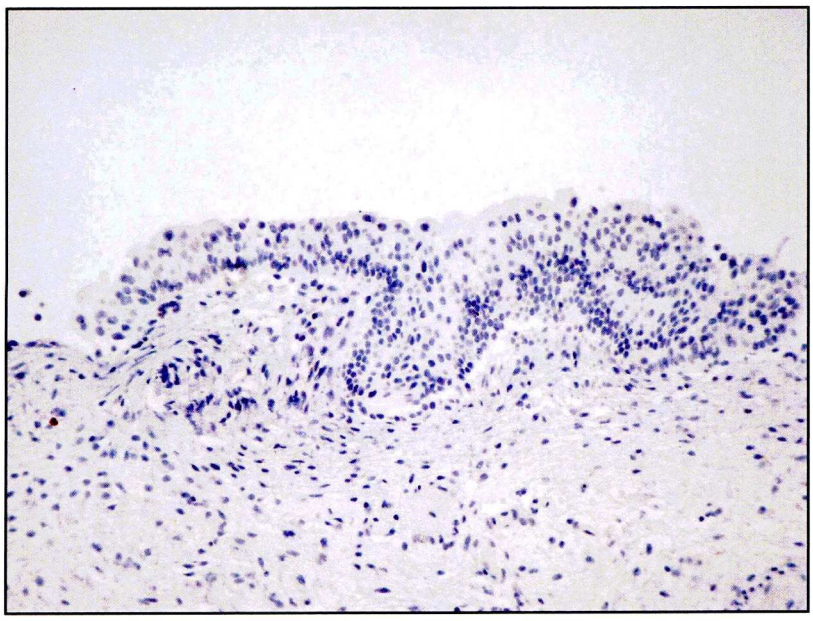


Figure 5.1: Early lesion with dysplastic morphological features but no proliferation identified by Ki=67 nuclear staining. (magnification x240)

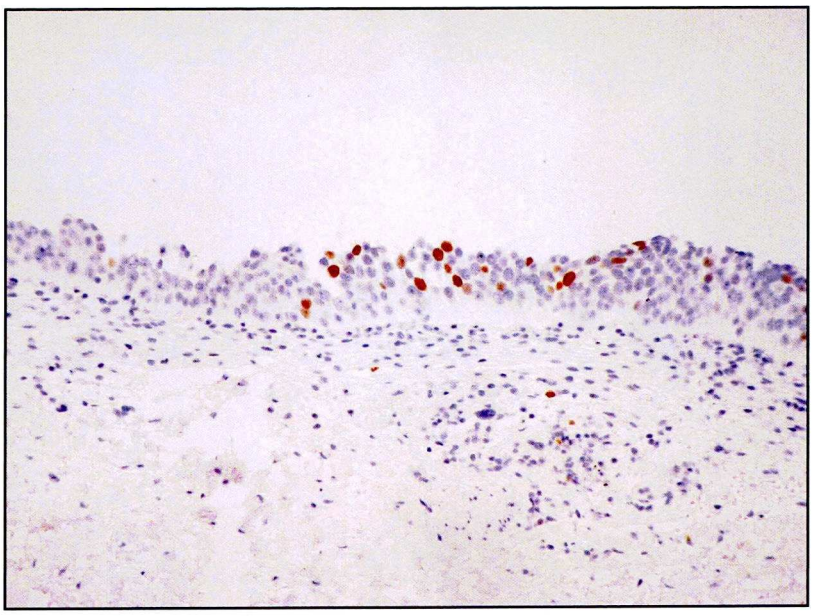


Figure 5.2: Flat dysplastic lesion (pTa) with focally increased proliferation (proliferation Group 2) (magnification x240)

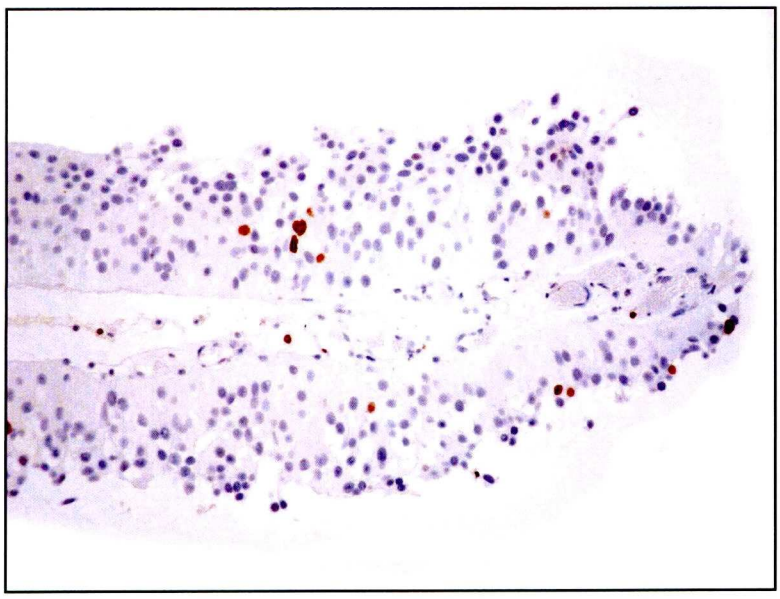


Figure 5.3: Grade II papillary lesion (pTa) with only occasional proliferating cells (proliferation Group 1) (magnification x240)

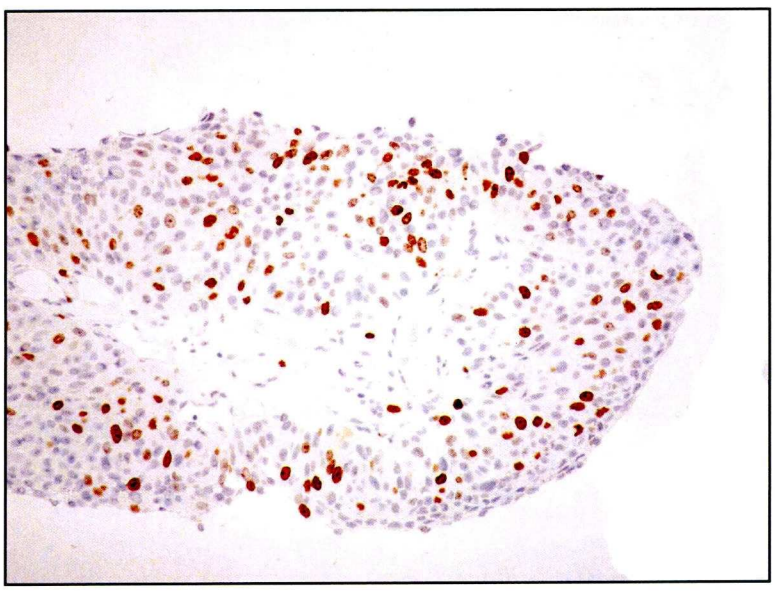


Figure 5.4: Grade II papillary lesion (pTa) with elevated proliferation (proliferation Group 3) (magnification x240)

	POSITIVE BASELINE SCORE	POSITIVE KI-67 INDEX	KI-67P	KI-67D
Normal	4/14	6/14	<0.01	0.22
Inflammation	7/9	8/9	0.01	0.23
Dysplasia	5/8	6/8	0.21	0.79
CIS/Superficial Cancer	35/37*	39/39	0.37	2.41
Muscle invasive Cancer	13/14*	13/16	0.08	1.96

* Three unavailable

Table 5.1: Staining Characteristics based on tumor staging

To compare the Ki-67p and Ki-67d indices, these were further grouped into the four ranges (0-0.1[1], 0.1-1[2], 1-2[3], 2+[4]). Using Spearman's test (r_s) there was a significant agreement between the indices used in comparison to the baseline scores (Ki-67p r_s =0.75, Ki-67d r_s =0.52). Ki-67p and Ki-67d were in almost total agreement (± 1) with the corresponding baseline score in 92% and 70% of the slides, respectively. The proliferation rates measured by Ki-67p and Ki-67d revealed a steady increase with stage. However, there was a decline in proliferation rate as some tumors became invasive (Figure 5.5 and 5.6). This was not evident with grading, worsening grade being associated with increased proliferation.

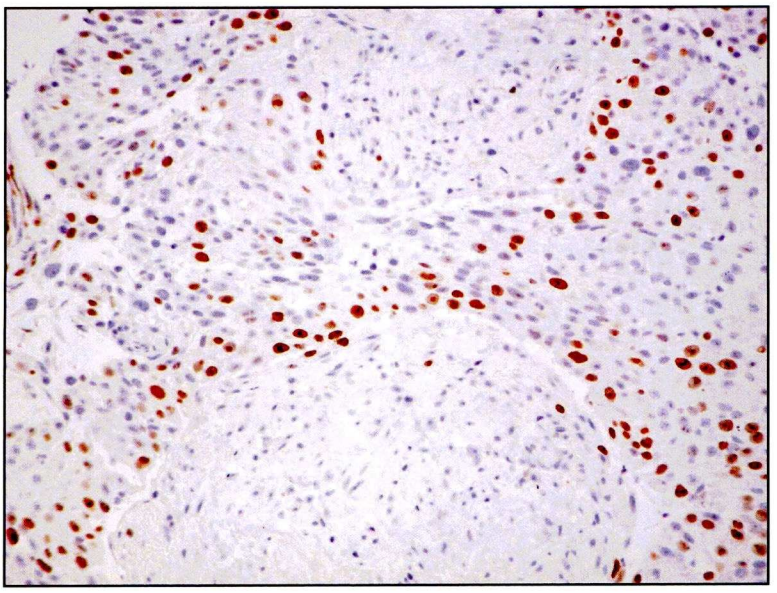


Figure 5.5: Muscle-invasive (Grade II, pT_2) bladder cancer with low-to-moderate cancer cell proliferation (Group 3). Proliferating cells are predominantly observed within the lymphocytic component. (magnification x240)

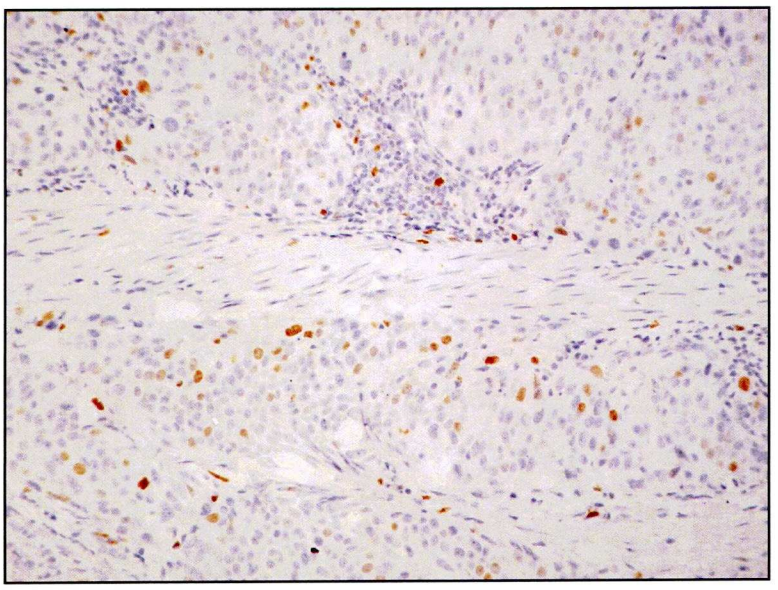


Figure 5.6: Muscle-invasive (Grade II, pT_2) bladder cancer with high cell proliferation (Group 3). (magnification x240)

More than two index readings were available for 10 patients to analyse disease progression (Table 5.2). Mean follow-up in these patients was 3.6 years (range: 6-132 months). Three of these 10 patients showed disease progression. No correlation was identified between the proliferation indices and disease progression. However, in this small group within the study population, a higher initial Ki-67 index indicated an unstable tumor-cell population. In the six patients with initial Ki-67d $\geq$0.005, Ki-67p >0.3 disease progression occurred in four (italics) and down staging of their cancer occurred in two (bold). The two in which their disease was down-staged had markedly high initial proliferation indices (Ki-67d>0.2, Ki-67p>4) while the four in which their disease progressed exhibited only moderately elevated indices (Ki-67d=0.005-0.03, Ki-67p=0.4-2.2). The disease was clinically non-progressive in four patients with lower tumor indices (Ki-67d<0.005, Ki-67p$\leq$0.1).

PT. NO	FOLLOW UP (YRS)	INITIAL SCORES					FINAL SCORES					PROGRESS
		YR	DIAGNOSIS	BASELINE	KI67 D	KI67 P	YR	DIAGNOSIS	BASELINE	KI67 D	KI67 P	
1	**3**	**99**	**G2pT1**	**3**	**.215**	**6.1**	**02**	**G2pTa**	**2**	**.246**	**1.63**	**No**
2	0.5	02	G2pTa	1	.004	0.1	02	G2pTa	2	.375	8.6	No
3	*2*	*00*	*G2pTa*	*3*	*.007*	*0.35*	*02*	*G3pT1*	*3*	*.051*	*2.09*	*Yes*
4	*11*	*91*	*G2pTa*	*2*	*.005*	*1.34*	*02*	*G3pT1*	*3*	*1.02*	*15.3*	*Yes*
5	5	96	G3pT2	2	0	0	01	G3pT1	3	0	0.12	No
6	*1*	*01*	*CIS*	*2*	*.03*	*2.16*	*02*	*G3pT1*	*2*	*0.008*	*0.6*	*Yes*
7	**2**	**97**	**G2pTa**	**3**	**.188**	**4.27**	**99**	**Dys**	**2**	**.021**	**0.21**	**No**
8	4	98	G2pT2	2	0	0	02	G3pT2	2	.017	0.74	No
9	7	95	G3pT1	1	.002	0.01	02	Infl	1	.003	0.04	No
10	*0.5*	*01*	*G3pT1*	*2*	*.034*	*1.47*	*01*	*G3pT3*	*2*	*.011*	*0.6*	*Yes*

Table 5.2: Progression and mean Ki-67 expression in 10 patients with more than two index biopsy specimens

5.7: Discussion

Ki-67 expression has been widely used to determine cellular proliferation rates in a range of malignant tissues and it has been shown to be an independent predictor of disease progression in the prostate [258] and in proliferative breast disease [267]. Other studies have confirmed a correlation between Ki-67 expression and bladder tumor stage and grade [269-272]. We have refined the analysis of tumor-cell proliferation in bladder neoplasia to define two new indices: Ki-67percent (Ki-67p) and Ki-67density (Ki-67d) that have been used to develop a semi-automated analysis of these parameters. In accordance with similar studies analysing Ki-67, we have demonstrated a correlation between Ki-67p and Ki-67d with tumor stage and grade. Studies on recurrence and Ki-67 indices suggested a higher labelling index in those with recurrent disease [269 271-273] and worse survival [274]. Although our study was unable to identify a relationship between Ki-67 expression and tumor recurrence, we showed a significant decline in cell proliferation rate in some tumors with progression to muscle invasive disease ($pT_1 \rightarrow pT_2$ transition). In a small subset, tumors with higher initial proliferation indices comprised a behaviorally unstable cell population with either progression or down-staging of disease. These tumors also tended to reduce Ki-67 expression over time. Lower initial Ki-67 indices were associated with static or indolent disease. The biological significance of these observations is that while low cell proliferation, identified by low Ki-67 expression, may indicate phenotypic indolence, very high Ki-67 levels signify genetic instability that, in other carcinomas may be associated with

good clinical prognosis [275 276]. It is possible that these cells are biologically incompetent and hence unable to survive or to progress to malignant neoplasms.

A cellular phenotype is the vectorial consequence of a number of constraints (gene expression, epigenetics, epithelial-stromal cross-talk etc.) that interact to produce cells with features that are collectively characteristic in appearance and behavior [277]. These are complex interactions between numerous processes that span several scales of time, involve a variety of components and are also determined by environmental location. This complexity has generated contrasting views on fluctuations in levels of gene expression and of their corresponding protein products [278 279]. Consequently, the phenotype of an individual cell, whether benign or malignant, is not static but potentially changing occurring whenever one of the constraints becomes modified. Simultaneous comparative gene-expression monitoring has been used to identify functional groups of genes whose patterns of co-regulation has provided the basis for separating bladder tumors according to microanatomical location [280]. That study revealed particular gene-expression profiles to characterize each pathological stage, suggesting the phenotype-modulating effects of environment. This current study has shown that proliferation of cells within individual urothelial cancers to be influenced by the environmental location, whether in the lamina propria (pT_1) or in smooth muscle (pT_2). These observations have significant implications for current concepts of the malignant phenotype, especially when understanding of the mode of action of therapeutic agents and the

consequences of their use. Thus, the steady state of a population of cells depends upon the interaction of several complex variables that include, *inter alia*, the rate of cell proliferation promoting expansion and the rate of apoptosis retarding expansion of that population as well as the metabolic competence of those cells (e.g. aerobic *vs* anaerobic respiration) with respect to constraints pertaining within the local environment at the time. It is not only gene expression that predicates the activity or competence of a cell but the relative amounts of protein translated from its transcribed mRNA and thereafter modulated by epigenetic factors. Our previous studies of estrogen receptors [281-283] emphasized a functional relationship between these proteins such that their *ratio* provides the regulatory control, rather than their absolute levels. While superficial bladder carcinomas with a low initial rate of cell proliferation may benefit from delayed chemotherapeutic modulation, a very high level of Ki-67 expression might indicate a possible unstable cell population with an ineffective impact on tumor progression and consequently a comparatively better prognosis than those with moderate cell proliferation.

The semi-automated system described in this study has enabled Ki-67 analysis produces objective and quantifiable data of greater accuracy than can be obtained subjectively. Significant correlation was identified between the baseline scores obtained subjectively and the semi-automated numerical indices. This study reported a better agreement between Ki-67p and baseline scores in comparison with the Ki-67d than could be explained by an observer analysing the entire field rather than restricting observations to the

carcinoma fields only. Conversely, fully automated image analysis is not currently able to distinguish between morphologically different areas, such as invasive cancers and stromal tissues. Therefore, any automated technique currently requires at least some level of operator guidance, at least to enable recognition of the field to be analysed, and an informed understanding of each tissue being assessed is currently necessary. Experienced operators are able to integrate, at a subliminal level, areas of importance that a computer is unable to distinguish. Nevertheless, computer-based image analysis provides objective numerical data available for comparative studies between serial biopsies over time, or with other parameters, such as expression of cell-cycle checkpoint proteins, to provide a robust assessment of the biological competence or progression of an individual bladder cancer.

In conclusion, the two parameters of Ki-67p and Ki-67d as defined in this study, correlate well statistically in bladder cancer. An increase in these indices correlated ($p<0.001$) with both tumor grade and invasion. There was also a significant agreement between the indices and baseline scores. Patients with initial low proliferation rates showed stable disease while moderately increased indices were associated with disease progression. However, those exhibiting very high initial proliferation indices appeared unstable with respect to risk of progression with some diseases appearing to undergo regression. As those tumours became muscle invasive ($pT_1 \rightarrow pT_2$ transition), proliferation indices declined. This observation could be explained by tissues with higher cellular proliferation rates having different biological and pathological constraints in locations where identical

regulatory criteria may not apply. Computer-assisted image analysis to quantify maximum cell proliferation according to Ki-67 expression in diagnostic biopsy specimens is a more accurate predictor of bladder cancer progression that could be employed routinely as one of the core parameters to determine individual bladder cancer management.

Chapter 6:

Biomarker expression pattern in Benign Urothelium and Non-Muscle Invasive Urothelial Carcinoma

A diagnosis of urothelial carcinoma is associated with a significant morbidity to the patient as well as a need for long term surveillance. In addition to the economic burden, this diagnosis often presents a therapeutic conundrum for the clinician. Approximately 80% of the newly diagnosed urothelial carcinoma present with non-muscle invasive or superficial carcinoma[185]. Patients with diagnosed urothelial carcinoma are treated with transurethral resection of the tumour. These often respond to further treatment with adjuvant chemotherapy or immunotherapy. The risk of recurrence in these patients is 70%[185]. Further need for treatment depends on the cystoscopic surveillance findings and histopathological staging; if recurrences occur. Despite an array of prognostic factors ranging from tumour size, primary stage and grade, to molecular analysis; the ability to assess and predict disease progression is still ambiguous.

Malignant transformation of normal cells occur either through expression of new genes (oncogenes) or the loss of the regulatory genes (tumour suppressor genes)[284]. The ability to detect cancer cells early and predict the nature and aggressiveness of the tumour population is limited at present to routine histopathological analysis. The optimal management requires the accurate assessment of the tumour biologic potential which is not entirely possible with just histological determination. Recent changes in the assessment and treatment of non-muscle invasive urothelial carcinoma has been based on categorizing this disease based on the various biological forms. This was conceptualized because apparently homogenous forms of non-muscle invasive urothelial cancer seem to behave differently in

response to treatment, suggesting that histologically similar disease might actually have different biological pathways.

6.2: Cell Cycle Regulatory Proteins

Studies have looked at alterations and aberrations in molecular pathways which govern urothelial tumourigenesis. Normal cellular proliferation occurs by a systematic progression through the various stages of the cell cycle, which also is the most common site of biomarker alteration in human urothelial carcinoma. The character of the cell is defined by how it responds to cellular stresses such as DNA damage. DNA damage can be purged either by DNA repair or by the initiation of apoptosis[285]. There is a consequent decreased risk of uncontrolled excess of abnormal cells and therefore a reduced risk of cancer formation. Deregulation of these cell-cycle control mechanisms is a common occurrence in malignant tissue transformation. The cell-cycle associated markers that have been analyzed in this work have been the G_1/ S phase transition modulators p53, $p21^{Waf1}$, p16 and Rb.

Cellular proliferation markers such as Ki-67, proliferating cell nuclear antigen (PCNA)[286], cell adhesion complex protein (α-catenin)[287] have been studied as prognostic markers in bladder cancer. The predictive value of the most promising marker in this group is Ki-67 which has been discussed separately in Chapter 5.

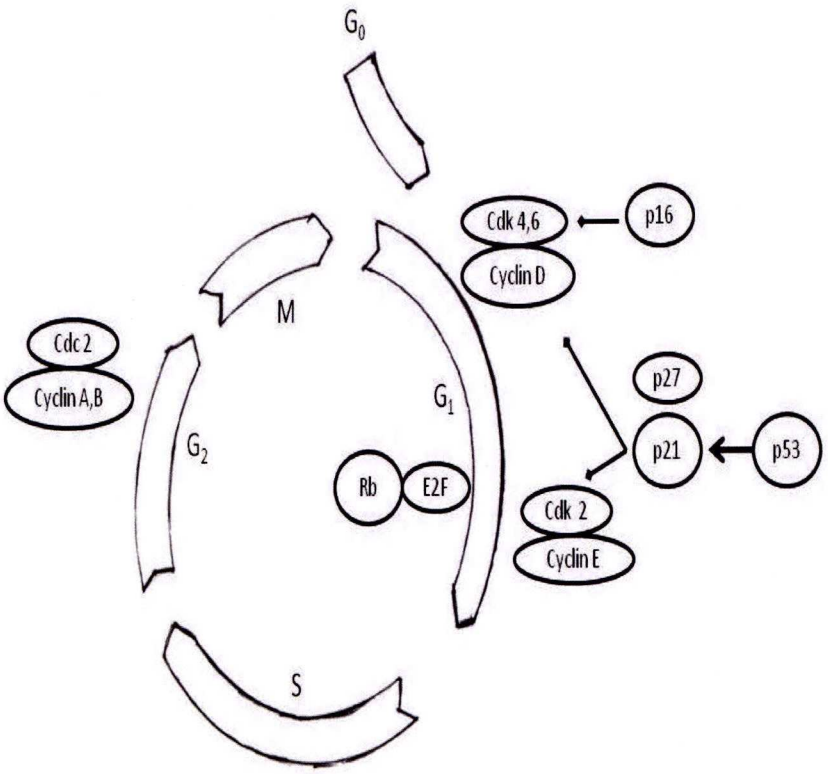


Figure 6.1: p16, p21, p27 and p53 inhibit the cell cycle by acting on the cyclin-dependant kinases

6.2.1: p53 protein

p53 tumour suppressor gene isolated to the chromosome 17p13, has a vital role in cell cycle regulation[288]. It is presumed that the wild type p53 may activate the transcription of inhibitory genes including down-regulation of the cell cycle and suppression of the initiation of DNA replication[225 289 290]. p53 is more involved in regulating and controlling the cell-cycle than in playing a direct role in the mechanism of cell cycle progression[291]. p53

mutations are the most common genetic alteration in malignancy[292]. This results in altered protein products with longer life compared to the wild type; accumulates in the cell with increased expression levels detected on immunohistochemical staining. It is an important prognostic factor for bladder cancer progression[139 293 294] with p53 accumulation associated with increased grade and stage of cancer, and poor clinical outcome[295-299]. In this study cohort, there was a highly significant gradation in p53 expression between the controls, early superficial disease and pT1 urothelial carcinoma. However, the data available regarding the role of p53 as a prognostic indicator in patients with non-muscle invasive tumours has been conflicting[141 300].

Zlotta *et al.*[129] showed significant p53 expression correlation with stage in superficial bladder cancer. Our study also revealed similar correlation in non-muscle invasive carcinoma (Figure 6.2). Sarkis *et al.*[141] studied a cohort of 43 patients with non-muscle invasive urothelial carcinoma; reporting tumour progression in 76% of the patients with >20% p53 expression while only 17% with <20% expression developing tumour progression. A strong p53 staining showed significant correlation with muscle invasive behavior and poor clinical outcome. In this study cohort, over 70% of patients who had progression to invasive disease had high initial p53 expression. Esrig *et al.*[139] found that patients with urinary bladder confined disease with p53-negative tumours tend to have a low rate of progression. However, they did not feel that this correlation was as robust as reported and suggested that the nuclear accumulation of p53 could occur in the absence of p53 gene

mutations. The p53 gene mutation or lack of in the presence of immunohistochemical detection of p53 protein could explain the disparity in assessing the prognostic benefit of p53 in urothelial cancer. Later studies have confirmed that p53 does have an important role as a biomarker[301 302]. Studies on p53 have found high levels of immune-reactivity in tumours with high propensity to recurr[294 303]. Moreover, some studies report an association between p53 over-expression and tumour recurrence also in non-muscle invasive disease[138 304 305].

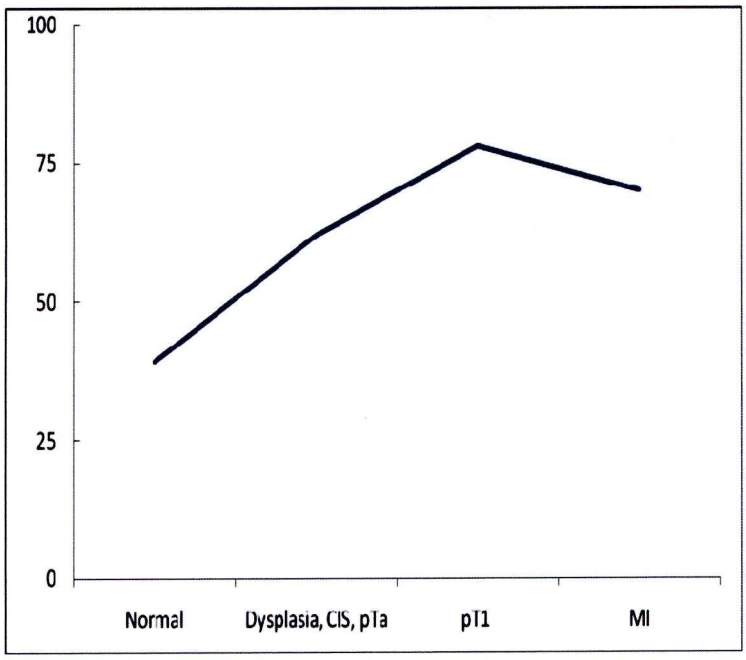


Figure 6.2: p53 expression (%) by tumour stage.

(MI-muscle invasive carcinoma)

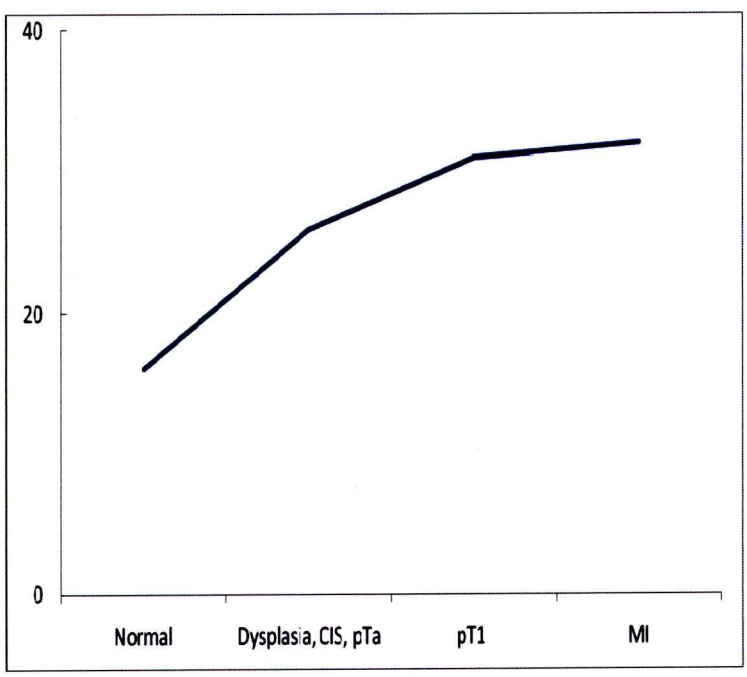


Figure 6.3: p21 expression levels (%) by tumour stage.
MI-muscle invasive carcinoma

6.2.2: p21 protein (WAF1/CIP1 gene)

p21 is a 21kD protein encoded by the WAF1/CIP1 gene. It is a potent inhibitor of cyclin dependant kinases which have a significant role at the G1-S transition for cell-cycle progression (Figure 6.1) thereby controlling cell proliferation and the ability to impede DNA replication[306 307]. It interacts with cytoplasmic proteins in cellular response to DNA damage and also has a role in cellular protection against apoptosis. p21 is a p53 downstream mediator of the antiproliferative function of the wild-type p53[306]. The ability of p53 to control the cell cycle is related to the ability of p53 to activate genes such as p21[129]. Studies[185 308] have shown that p53 exerts its effects on the cell cycle through controlling $p21^{(WAF1/CIP1)}$ expression. p53 alterations lead to loss of p21 protein expression leading to unregulated cell growth. The overall positive rate was 25% with 28% positivity in non-muscle invasive disease.

Stein *et al.*[185] reported the strongest association between p21 status and tumour progression in patients with organ confined disease ($\leq$pT3a). Loss of p21 expression was strongly associated with increased probability of recurrence and decreased survival[185]. Despite p53 being a significant brake-point in the cell cycle, maintenance of p21 helps protect cells from the detrimental effects of p53 alterations[122]. This could be because the effects of p21 on the cell-cycle maybe through p53 independent pathways[123 175]. They also opine that patients with tumours that have altered p53 and lost p21 have a poorer prognosis. However, we were unable to show any significant

association between tumour stage and p21 expression. In this study cohort, there was a marked increase in p21 expression within dysplasia, CIS, pTa disease. Loss of p21 expression was seen in pT1 stage. Based on our data, we found preservation of p21 expression in early NMI. Loss of p21 reflects worsening grade over pT1 disease and muscle invasive disease (Figure 6.3)

In the normal controls, there was low p21 expression suggesting that the increased p21 expression suggests malignant transformation. This agrees with Korkolopoulou *et al.* who reported expression of p21 early on in tumourigenesis[309]. However, in our cohort, presence of established urothelial carcinoma, loss of p21 implies aggressive tumour; which suggests that non-muscle invasive and muscle invasive urothelial cancer could be two differing disease entities.

6.2.3: *Rb* protein (Retinoblastoma gene)

Rb protein in the hypophosphorylated state acts as a tumour suppressor inhibiting cell cycle progression at the G_1/S restriction point. Accumulation of the hypo-phosphorylated pRb blocks E2F dependant transcription leading to cell-cycle arrest[230]. Phosphorylation of pRb renders it active; activating factors like cyclins which increase cellular proliferation rates. Alteration of the Rb gene expression has been found in different types of tumours including bladder carcinoma[148 310]. Hypophosphorylated Rb acts at the G1-S

check point binding to a number of cellular proteins including E2F (a transcription factor)[311 312], thereby inhibiting cell-cycle progression.

Rb gene inactivation is thought to be a critical step in bladder cancer progression[185]. Kubota *et al.*[313] suggest that Rb loss is involved in tumour initiation. In this study cohort, despite not reaching significance, there occurred Rb loss in non-muscle invasive urothelial carcinoma. Most studies have reported dual inactivation of p53 and pRb suggesting that this occurs in oncogenesis because pRb deficiency results in uncontrolled cellular proliferation; which with defective p53 does not result in apoptosis[314 315]. Studies have also reported a role for p16 as a modulator for Rb expression levels[316].

6.2.4: p16 protein (CDKN2a gene)

The gene for p16 (CDKN2a) is located on the 9p21 gene. It is a crucial inhibitor of the cyclin-dependant kinase complex and it is inactivated to permit progression through the G1/S cell-cycle check points[227 228]. Up-regulation of the cell cycling occurs with the loss of p16 function and subsequent loss of Rb tumour suppressor function[227-229]. Santos *et al.*[317] suggested that the loss of p16 staining is an early genetic event in bladder carcinogenesis. It has been reported to be more common in pT1 than in muscle invasive disease[117 318]. This loss of protein expression was not

associated with an increased risk of recurrence or progression[245 317]. However, other studies have reported variable results[319-321].

Nakazawa *et al.*[322] studied p16 expression analysis finding weak expression in non-neoplastic lesions. They also found a higher incidence of p16 over-expression in non-infiltrating urothelial carcinoma. Our study showed low levels of expression in normal cells; significant increased expression in non-muscle invasive carcinoma in comparison to the benign bladder tissue. There were similar levels of high expression between the early superficial NMI carcinoma group (dysplasia, CIS and pTa disease) and pT1 urothelial carcinoma.

The variable reports could be explained by some degree of cytoplasmic staining in all p16 positive cells[151]. This background cytoplasmic staining was considered normal p16 staining as it was seen only in the tumour cells, considered specific for p16.

6.3: Apoptosis regulators

There are several noxious stimuli which initiate and propagate the process of cellular death. This is brought about by the disruption of the dynamic equilibrium between apoptotic cell death and cell survival by alterations in gene products regulating cell survival such as p53 (discussed earlier), Bcl-2 and other homologues of the bcl-2 family such as Bax[323 324] and bcl-X[199]. The apoptotic process is initiated by pathways involving either the activation of death receptors on the cell surface (extrinsic pathway) or mediated by mitochondria (intrinsic pathway)[301]. The final common pathway leads to the activation of cysteine caspases that initiate and effect irreversible cellular changes[301].

6.3.1: Bcl-2 protein

The bcl-2 protooncogene anti-apoptotic action is by regulating a downstream event in a final common pathway leading to apoptotic cell death[208]. The bcl-2 protein is involved in the intrinsic pathway and controls mitochondrial membrane permeability and inhibits caspase activation[325]. Increased Bcl-2 expression has been associated with decreased tumour-free survival in G3pT1 disease[326]. In this study cohort of 301 index bladder biopsies, there was significant loss of bcl-2 expression in the non-muscle invasive cancers (Figure 6.4), both pTa and pT1 disease with no significant difference between these two groups. In total, there were only 14 Bcl-2

positive specimens (8%) from a total of 186 index bladder biopsies with bladder malignancy (Table 6.1). Other studies[199 327] also report similar low Bcl-2 (5%-7%) detection rates in urothelial transitional cell carcinoma.

Grade & Stage	Positive staining (n)	% of cases
Normal	11	65%
Chronic Inflammation/ NAD*	25	33%
Dysplasia → G_1pTa	10	14%
G2pTa	1	2%
G1/2pT1	1	5%
G3pTa	0	0%
G3pT1	2	7%

*NAD-No abnormality detected

Table 6.1: Percentage Bcl-2 expression by Grade and Stage

Sub-group analysis of the cancer group did not show any variation in the very low Bcl-2 expression levels in histological malignant bladder tissue. Only 8% of pTa (including dysplasia and CIS) tumours and 6% of pT_1 tumours had positive Bcl-2 expression. Therefore, I could not opine on the suggestion that persistence of Bcl-2 expression in bladder malignancy implied better prognosis in various stages of bladder malignancy[328].

In this study, there are higher levels of bcl-2 expression within the true normals (65%) and in the inflammatory biopsies (33%). This is in contrast to published literature where the expression in "normal urothelial cells" has also been reported to be low[199 327]. However, the histological normal tissue

considered as controls in these studies were either from patients with cancer (renal and bladder)[199] or not described [327]. I do not feel these tissue samples are biologically normal urothelium and suggest that these have differing biological characteristics in comparison to normal urothelial tissue from patients with no suspicion or history of urothelial malignancy. This is discussed later in Section 6.9.

In contrast, malignant urothelium had low bcl-2 expression (0-14%) suggesting an association of bcl-2 loss with tumourigenesis. Extrapolating these findings imply that sparing of bcl-2 expression in malignant tissue could be of prognostic benefit. The evidence is far from robust in this regard and needs further studies into the genetic background and the role of other tumour markers in bladder cancer apoptosis.

6.3.2: Bcl-X_L

Bcl-X is an anti-apoptotic gene, a homologue of the bcl-2 gene family. It demonstrated different expression patterns and was thought to regulate cell cycle at different stages of cell differentiation than bcl-2[199]. Bcl-X encodes for two proteins. Bcl-X_L is the longer protein transcript of bcl-X gene suppressing cell death with the shorter bcl-X_s promoting apoptosis[208].

Bcl-X has been shown to be over-expressed in malignant bladder neoplasms and correlated with stage[329]. In our study cohort, normal urothelium expressed very low levels of bcl-X_L which continued to be maintained in the early carcinoma. pT_1 disease is associated with marked over-expression. We had smaller numbers of bcl-X_L which could explain why the data did not reach statistical significance.

6.4: ErbB-2 Oncogene

This oncogene is located on chromosome 17q21, a member of the human epidermal growth factor receptor family[330]. ErbB-2 (Figure 6.5) protein has an intra- and extra-cellular domain with tyrosine kinase activity involved in signal transduction of cell growth[331 332]. Amplification of the gene has been reported in over 30% of women with breast cancer with consequent protein receptor over-expression; significantly associated with tumour aggressiveness and poor survival[333 334]. It has also been described in other solid tumours such as colon, lung, ovary, stomach and thyroid[335].

In bladder cancer, erbb-2 amplification has been associated with almost 20% of the advanced and high-grade tumours[336]; a very frequent amplification in bladder cancer[335]. Increased erbB-2 expression in bladder cancer has been found to correlate with tumour recurrence[337], aggressive stage[338], and poor prognosis[338]. Lipponen et al. [339] suggested an association

of moderate/ severe erbb-2 over-expression with a more aggressive tumour type. However, in low malignant potential papillary tumours, studies have shown no correlation between protein expression and stage or grade[304] which is in compatible with our results. In our cohort, there was no difference in erbb-2 expression levels between normal urothelium and NMI. Erbb-2 does not appear to have a role in early urothelial cancer.

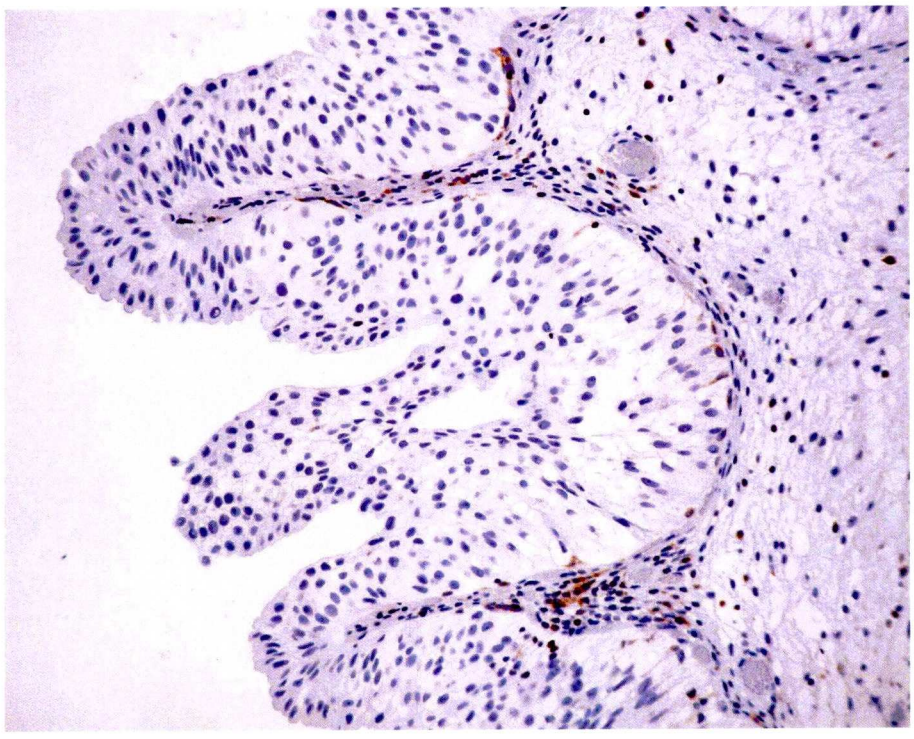


Figure 6.4: Bcl-2 expression loss in urothelial carcinoma

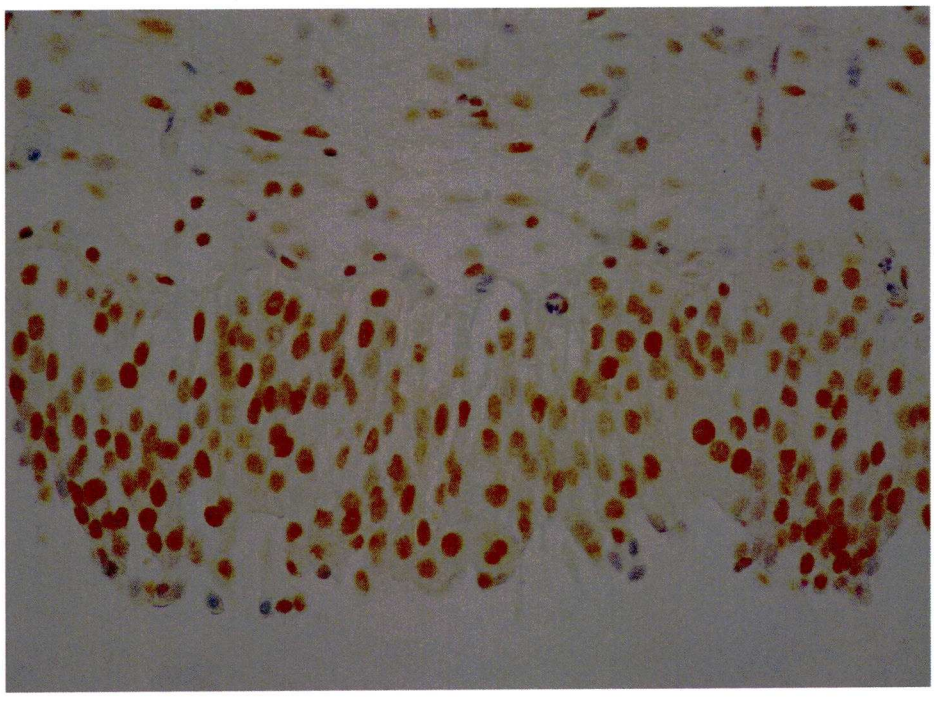


Figure 6.5: erbB-2 expression in normal bladder urothelium

6.5: PTEN tumour suppressor gene

This tumour suppressor gene mapped to the 10q locus has been suggested to play role urothelial cancer[340]. PTEN mutations have been identified in various cancers[340] including prostate carcinoma[341] and in the urinary bladder[233 342]. Wang et al.[340] suggest that loss of PTEN expression could be a late event in bladder cancer. We did not find any significant variation between the urothelial tumour stages or the benign group.

6.6: Oestrogen Receptors:

ER α and ER β are the two major oestrogen receptor subtypes which modulate the effect of oestrogen on cell proliferation and survival. It is especially important in tumour-prone tissues which are responsive to hormones such as the uterus and breast[343] which have higher receptor levels. Studies have shown over-expression of ER α in breast cancer with down regulation of ER β[344 345]. ER β over expression resulted in reduced cell proliferation and with a concomitant stunting of the cell-cycle[344 345]. Based on these studies, Helguero et al.[345] raised the hypothesis that ER β has a significant role in inhibiting ER α activity and the loss of ER β is associated with the development of cancer[345].

Most studies on oestrogen receptors in urothelial cancer have reported ER β expression loss with malignant change resulting in an increased ER α /ER β

ratio[346-348]. High levels of ER α have been found in a large number of human bladder cancers[239]. Teng et al.[343] also found significantly higher levels of ER α in malignant urothelial cells but with similar levels of ER β expression within both tumour and normal cells.

In this study cohort, all tissue showed no ER α expression, neither in benign urothelial tissue nor in different stages of urothelial NMI carcinoma. However, normal urothelium showed a high ER β expression (42%) with subsequent loss in carcinogenesis with levels dropping to 20% (Table 6.2).

This study lends credence to the Helguero hypothesis[345]. The results suggest that it is probably the ER β receptor that plays a major role in urothelial carcinoma and not the ER-α receptor as reported so far.

		Benign urothelium	Dysplasia, CIS, pTa	pT1 disease
ER α*	n	2	67	24
	%	0	0	0
ER β	n	19	123	50
	%	42	21	20

* all ER α stained tissue were negative

Table 6.2: Oestrogen receptor expression Vs tumour stage

6.7: Heat-shock protein 27 (hsp-27)

Heat shock proteins (HSPs) are highly conserved chaperone proteins expressed in cells usually in response to stress. Expression levels are often increased in a wide range of cancers. In this family, hsp-27 is a cytoplasmic protein which is present within normal and malignant cells. It was initially discovered as an oestrogen-modulated protein in breast cancer[349] and then found to contribute to apoptosis[350]. Hsp-27 over-modulation has been shown to be predictive of death from prostate cancer[351], subsequent development of breast malignancy from early proliferative breast lesions[352] and associated with grade in cervical neoplasia[353].

In this study, 254 index bladder specimen stained with hsp-27 were available for assessment with very high levels of (>85%) positive expression seen.

		Benign urothelium	Dysplasia, CIS, pTa	pT1 disease
Hsp-27	n	13	94	47
	%	93	90	87

Table 6.3: Extent of Hsp-27 expression Vs tumour stage

The only similar study in literature showed no significance in bladder cancer[354]. However, there occurred positive staining within the lamina propriae of histologically pTa tumours raising the possibility that these biopsies are showing signs of early lamina propriae invasion (Figure 6.6). However, the numbers were too small for meaningful analysis but could play a role in assessing stage migration.

6.8: Flat (Pre-) neoplastic lesions

One of the caveats of this study is the grouping of three disease entities under one umbrella. Dysplasia, carcinoma-insitu and pTa tumour were grouped together for analysis purposes. The molecular biology of these groups are similar but probably not identical. The histopathological diagnosis of these three entities is difficult with significant variability among histopathologists regarding what constitutes a particular diagnosis. This is because in dysplasia, the degree of architectural frailty is not as prominent as seen in carcinoma in-situ and even more so in pTa disease; despite sharing many similar morphological and genetic features. The cellular changes include marked nuclear enlargement, nuclear hyperchromasia, increased nuclear/ cytoplasmic ratio and some nucleolar abnormailities. Inter-observer variation and the lack of uniform definition have made differential reporting especially of dysplasia and carcinoma insitu difficult and often confusing. The same problems persist in the diagnosis of pTa disease.

Urothelial neoplasms are multifocal with a high rate of recurrence. The latest theory on urothelial carcinogenesis is the field defect theory suggesting that triggers effect genetically altered cells which become the source of polyclonal tumours[111 355]. Hodges et al.[356] postulate that based on the molecular alterations, that flat urothelial lesions comprise a wide morphological spectrum from reactive atypia to carcinoma insitu.

6.9: No abnormality detected/ Inflammation with no atypia

Molecular studies have suggested instability in normal urothelium in bladders with known or a history of urothelial carcinoma[356]. This again supports the concept of field changes in bladder urothelial cancer. In our study cohort (Figure 6.7), there were significant differences in bcl-2 ($p=0.017$), PTEN ($p=0.024$) and in erBB-2 ($p=0.011$) expression levels between the two groups, true normal and those with no histological abnormality but with a history of urothelial malignancy. Studies which use histologically benign urothelial tissue as control do not take into account these potential field changes confounding results. This study cohort offers evidence suggesting that patients with a history of urothelial neoplasia would always have a degree of urothelial instability and should not be considered to be normal. However, the molecular pathways in this distinct sub-group (histologically normal/ biomarker abnormal) need to be understood as probably a separate pathway and should not just be attributed to urothelial carcinogenesis associated field changes.

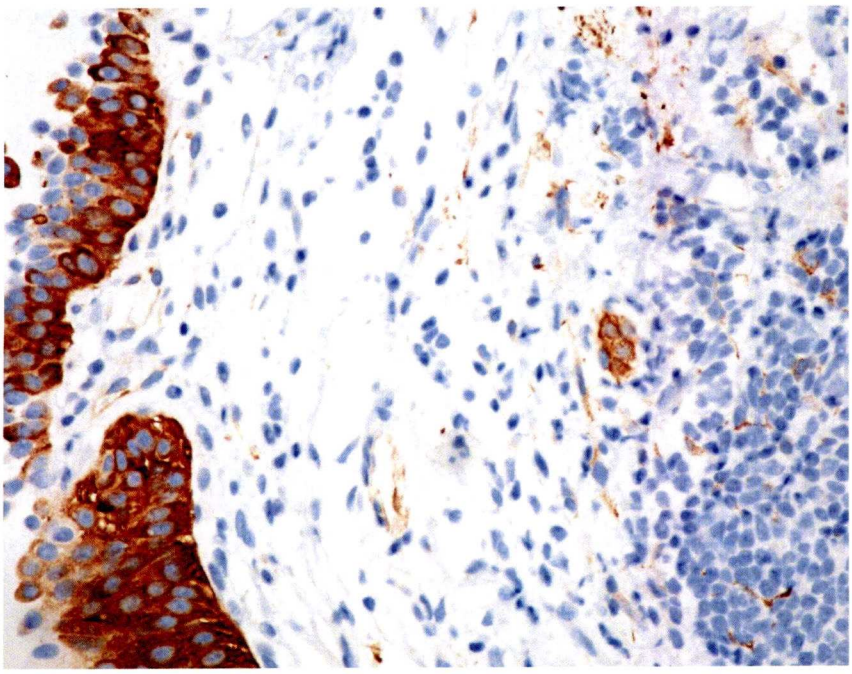


Figure 6.6: Clusters of invasive urothelial carcinoma within submucosa identified by strong Hsp-27 expression (Discriminating pTa from pT1)

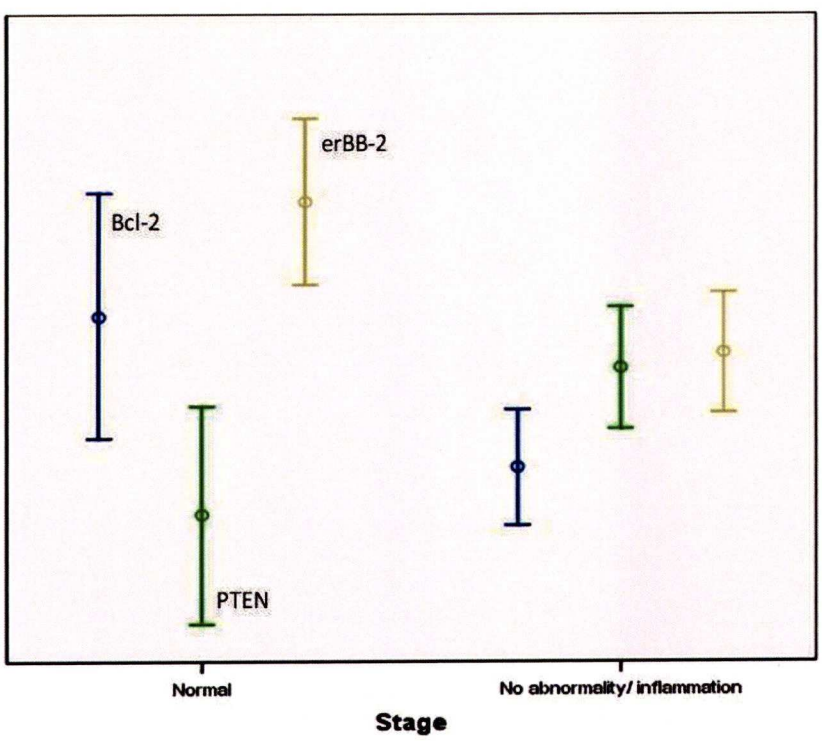


Figure 6.7: Biomarker variation between Normal urothelium and other non-malignant urothelium (inflammation/ history of urothelial malignancy)

Chapter 7:

Biomarker expression pattern in

Muscle Invasive Urothelial Carcinoma

The majority of patients with urothelial carcinoma have a non-muscle invasive tumour (pTa-pT_1 stage) at diagnosis. However, between 10% and 30% of the patients diagnosed with urothelial carcinoma have muscle invasive disease at diagnosis[119]. 70% of the patients with non-muscle invasive tumour recur after intial resection, and upto a fifth of these patients develop invasive urothelial carcinoma [119]. Urothelial carcinoma patients with muscle invasive disease ($\geq pT_2$) are at higher risks of recurrence and subsequent death from the disease. Treatment has to be aggressive; this is either surgery in the form of radical cystectomy for T_2, T_3 stages of the disease or chemotherapy/ radiotherapy in more advanced disease such as T_4 urothelial carcinoma, nodal or metastatic disease. Despite aggressive treatment, these patients have a high morbidity and mortality. Conventional histopathological assessment does not accurately predict the behavior of the urothelial carcinoma. Therefore, the therapeutic protocols have now centered on the need to identify the subset of urothelial carcinoma patients who are likely to progress, also those with a high propensity to recur and the tumours which could be resistant/ respond poorly to local treatment.

Indirect epidemiological evidence indicates that only a small number of patients with invasive cancer had a history of non-muscle invasive carcinoma[357 358]. However, Volante *et al.*[359] suggest that progression of urothelial carcinoma can occur from non-muscle invasive (superficial) to muscle-invasive urothelial carcinoma through accumulated genetic alterations. This is the most important challenge as the patients who develop muscle invasive tumour from primary non-muscle invasive tumour fare

worse than patients who have had a primary diagnosis of muscle invasive tumour [360]. Scientists have attempted to define tumourigenesis pathways so as to better understand urothelial carcinoma with the development of modern technologies such as immunohistochemistry. However, the process of malignant transformation of urothelium has been associated with various molecular pathways; the identification of which will help identify prognostic pathway-specific signatures. In this chapter, the focus is limited to the development and transformation to muscle-invasive urothelial carcinoma which I have tried to characterize with the help of the panel of markers. The significant markers involved in the development of/ transformation to muscle invasive tumour are discussed below.

7.1: Cell Cycle Regulatory Proteins:

Cell-cycle regulators are the most studied of the various biomarker groups in the development of muscle invasive urothelial tumour. Unregulated cellular proliferation is a cardinal dysfunction in carcinomatous transformation of urothelial cells. The normal cell-cycle is controlled by various cell cycle check-point regulators such as p53, Rb, p21 and p16. In this chapter, we report the salient biomarkers that have shown to be significant in the diagnosis of muscle invasive urothelial tumour or the progression to muscle invasive urothelial tumour.

7.1.1: p53

The prognostic value of p53 in muscle invasive urothelial tumour has been studied by various groups. Esrig *et al*[139] evaluated more than 250 patients who underwent radical cystectomy. They reported that p53 over-expression was associated with higher risk of disease progression and reduced overall survival irrespective of stage. They felt that p53 could influence selection of patients for adjuvant treatment. Patients with p53- positive urothelial carcinoma including those with superficially invasive disease have been reported to have a poor prognosis[139 141]. Sarkis *et al.*[141] reported poor clinical outcome (muscle invasive behavior) with strong p53 expression in a cohort of non-muscle invasive tumour patients. This study cohort also showed a marked loss in p53 expression in the muscle invasive cancer but with no prognostic significance associated with p53 expression. This is in agreement with Drager *et al.*[361] in their metaanalysis reporting no prognostic benefit with p53 in muscle invasive urothelial tumour.

The data suggests that there is a double-hit mechanism in the p53 associated tumourigenesis. This study shows low levels of expression in the controls with significant differential p53 positivity with non-muscle invasive disease and more so in pT_1 disease. Tiguert *et al.*[362] have also reported on the value of p53 immunostaining in predicting progression in T_1 disease. I found that the comparative p53 expression is less in muscle invasive disease suggesting that there is a second event with late loss of p53 implying a

poorer prognosis and aggressive character in agreement with the studies discussed above.

Studies have also assessed the effect of intravesical immunotherapy with Bacillus Calmette-Guerin (BCG). p53 negativity has been proven to herald progression in patients with recurrent tumours who have failed BCG treatment[363]. We have not been able to analyze the effect of intravesical chemotherapy/ immunotherapy as the number of patients who had undergone these treatments was small.

7.1.2: pRb (retinoblastoma gene)

Rb inactivation is considered a critical step in urothelial tumour progression. Rb susceptibility gene acts as a negative cell-cycle regulator by sequestering members of the E2F family of transcription factors which repress apoptosis. Studies have shown that higher grade and stage bladder tumours exhibit more Rb alterations[152 364]. Rb loss was significantly associated with shorter five year survival[148 149] in muscle invasive urothelial tumour compared to those who maintained Rb expression. However, other studies have failed to show a correlation between low pRb staining and recurrence or progression[317 152]. In this cohort, there was a no significant difference between the levels of Rb expression once malignant change had been established. Moreover, alterations in p53 and pRb may act in a synergistic

fashion to promote tumour progression[365]. This is common in locally advanced urothelial tumour and has a poor prognosis[139]

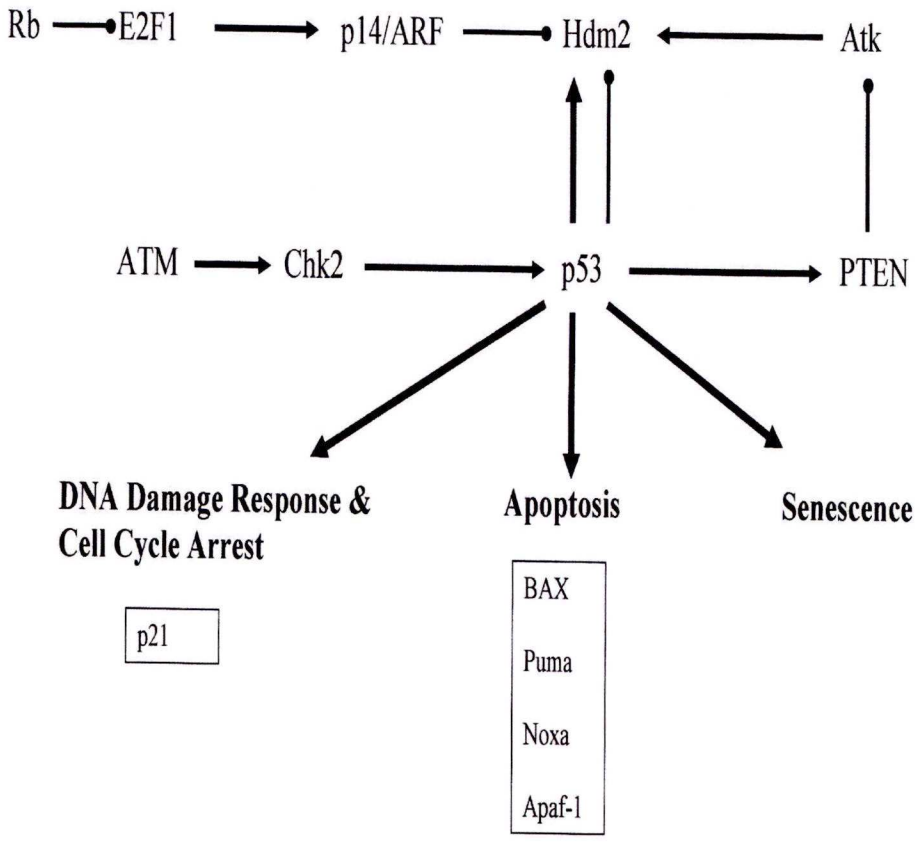


Figure 7.1: Cross talk between signaling pathways. Rb, p53 and PTEN[366]

7.1.3: p21

In this study cohort of 220 index bladder biopsies, p21 immuno-positivity occurred in 25% (55 patients) of the bladder tumours with 32% (6 patients) positivity in muscle invasive tumours. This is similar to published literature which report rates of 33% and 64% positivity[122 367 368]. Most studies on p21 expression have studied it in tandem with p53. Stein *et al.* evaluated 101

patients who underwent radical cystectomy for invasive bladder carcinoma and reported significantly increased probability of recurrence and significantly reduced overall survival[122]. In this study, p21 was the only independent predictor of disease progression in men with bladder tumours that had altered p53. Stein *et al.* suggests that patients with p53 altered tumours that loose p21 expression have a poor prognosis and should be considered for adjuvant treatment[122]. I did not find a significant difference in p21 expression levels based on stage. However, despite no correlation, 32% of the muscle invasive tumours were positive for p21 expression compared to only 16% in the normal tissue suggesting that p21 positivity might be associated with tumourigenesis. Figure 7.1 shows a possible pathway for p21 (interchangeable with p27).

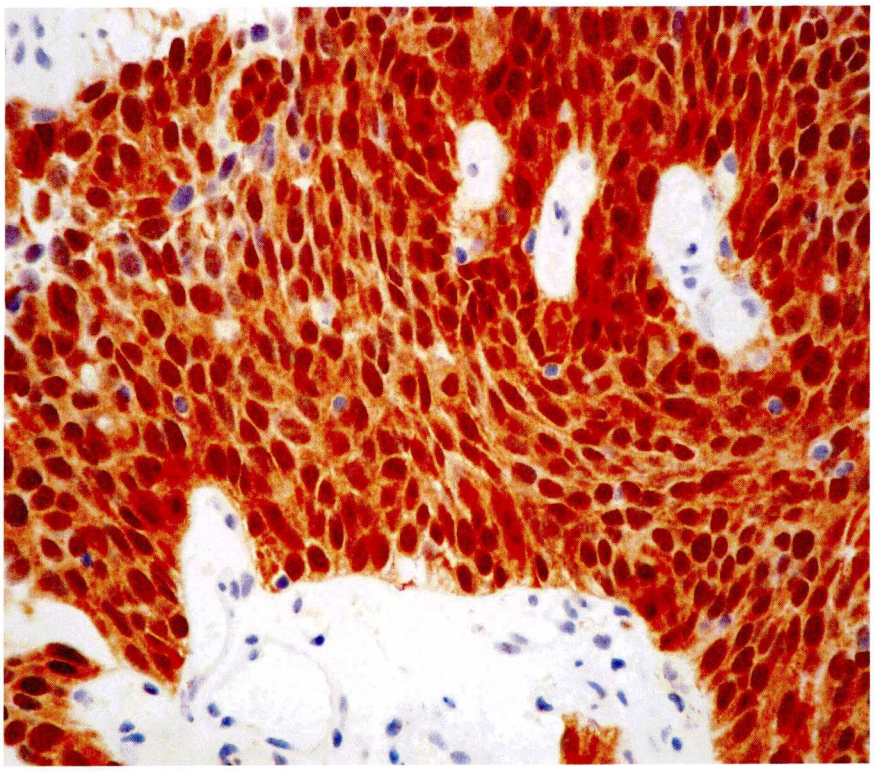


Figure 7.2: p 16 expression in muscle-invasive urothelial carcinoma

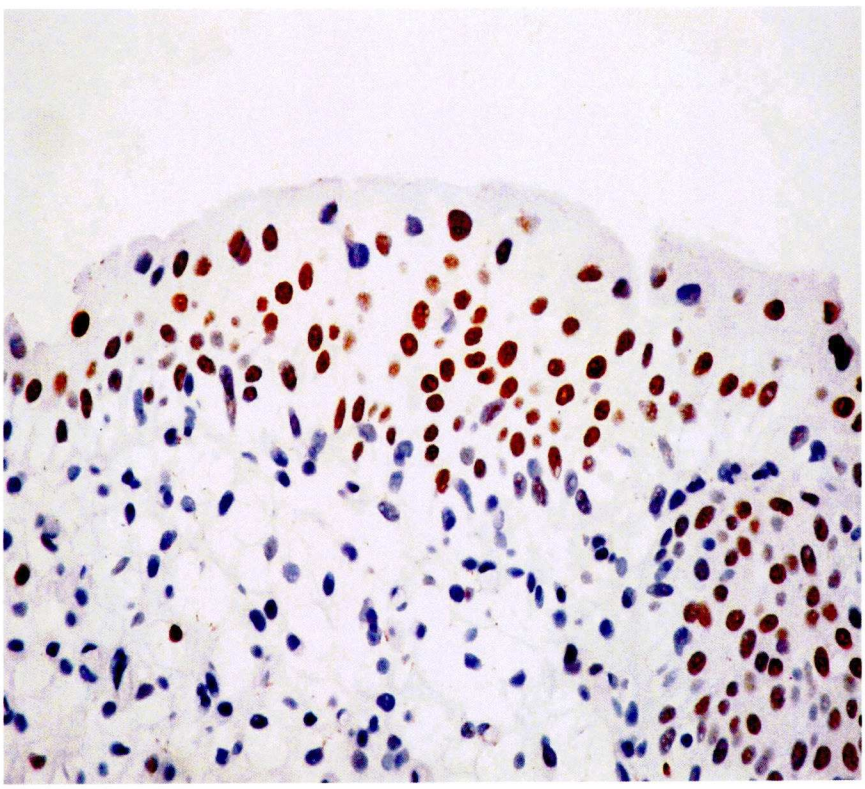


Figure 7.3: p 21 expression in muscle-invasive urothelial carcinoma

7.2: Apoptosis modulators

Apoptotic cell death is as important as cellular proliferation in maintaining a dynamic equilibrium. Therefore the effect of apoptotic markers also play an important role in the diagnosis, recurrence and progression of urothelial carcinoma.

7.2.1: Bcl-2 and Bcl-X_L

Bcl-2 protein has the ability to block apoptosis and the wild-type function of the p53 gene. We found high levels of bcl-2 expression in normal tissue. Uniform loss of expression was seen through all stages of urothelial cancer through to muscle invasive urothelial tumour. Glick et al.[369] found that only 24% of the invasive bladder tumours expressed Bcl-2 protein. There was a significant correlation with Bcl-2 absence and stage.

Bcl-X_L mediated micro-environmental signals have been reported in B-cell leukemia to reduce sensitivity to chemotherapy[370]. However the expression levels were very low in the bladder biopsies in our study thereby failing to enable any meaningful assessment. In contrast to Kirsh et al.[199], we found that muscle invasive tumour had significant over expression of Bcl-X_L.

		Benign urothelium	Non muscle invasive Tumour	Muscle invasive Tumour
Bcl-2	n	17	182	27
	%	65%	8%	7%
Bcl-X_L	n	2	82	12
	%	0%	4%	25%

Table 7.1: Extent of Bcl2 & Bcl- X_L expression Vs tumour stage

7.3: Oncogenes: erbB-2

erbB-2 is one of the most frequently amplified oncogenes in urothelial cancer. In urothelial cancer, it predominantly is expressed in advanced and high-grade tumours[336]. The expression of erbB-2 in muscle invasive urothelial carcinoma has been reported to range between 23% and 80%[337] [371-373]. We had similar results with over expression in 62% of these cases. erbB-2 expression has been reported to be associated with worsening stage[338] and decreased survival[338 374 375]. erbB-2 levels were high in the normal bladder tissue (90%) which was maintained in non-muscle invasive urothelial carcinoma with over-expression levels of 84% . However, there was a significant loss of erbB-2 with tumour invasion.

Most studies on erbB-2[339 376 377] have reported similar results suggesting that anti-erbB-2 treatment may have a role in muscle invasive urothelial carcinoma. Bolenz et al.[378] suggest that the assessment of erbB-2 status can

be used to identify patients at high risk of disease progression who would benefit from adjuvant erbB-2 targeted mono- or combined therapy after radical cystectomy.

7.4: Oestrogen Receptor β (ER β)

ER expression in muscle invasive urothelial carcinoma is a poorly understood entity. ER α staining is absent in all urothelial tissues. There was a complete absence of ER α expression across all disease stages and the benign urothelium. ER β loss significantly heralds onset of malignancy and is almost completely lost in muscle invasive urothelial carcinoma. This is in contrast to Kaufmann et al.[239] who found higher number of positive ERs in invasive tumours in comparison with non-muscle invasive urothelial carcinoma. Based on our observations, ER β appears to have a cytoprotective effect with loss of ER β expression implying carcinogenic transformation.

		Benign urothelium	Non muscle invasive	Muscle invasive
ER α*	n	2	91	12
	%	0%	0%	0%
ER β	n	19	173	28
	%	42%	21%	4%

* all ER α stained tissue were negative

Table 7.2: Extent of Oestrogen receptor expression Vs tumour stage

7.5: Disease progression: Non-Muscle Invasive urothelial carcinoma to muscle-invasive urothelial carcinoma

Rb, p21, PTEN and Hsp-27

Rb gene inactivation has been discussed in the previous Chapter. Studies have observed Rb mutations in low-grade non-muscle invasive urothelial carcinoma as well as high-grade muscle invasive urothelial carcinoma [143]. This ambiguity has been explained to a certain extent by the lack of concordance between Rb nuclear immunoreactivity and the presence of a functional protein. The use of Rb as an independent marker has therefore been limited. However, in this study, non-muscle invasive urothelial carcinoma which progressed had significantly ($p<0.005$) higher levels RB expression. This defined sub-group of patients also showed significantly higher levels of p21, PTEN expression and under-expression of hsp-27.

These three closely related cell-cycle proteins suggest the possibility of a specific gate/ check-point in urothelial carcinoma transformation from non-muscle invasive urothelial carcinoma to muscle invasive carcinoma. I have tried to elucidate the reasons behind these findings. The main restriction controls in the cell-cycle are mediated by cyclins[379]. The D-type cyclins (mainly D1) accumulate in the nucleus in the G_1 phase[380]. PTEN prevents cyclin D1 nuclear localization[381]. Chung *et al.* suggest that nuclear PTEN – mediated down-regulation of cyclin-D1 transcription may modulate G_0-G_1 cell cycle arrest[382]. Sherr[383] in his treatise on cancer cell cycles has

elucidated these pathways. He points out that the D-type cyclins; being unstable, accumulation depends upon persistent mitogenic signalling. This in turn triggers the Rb phosphorylation resulting in the stunting of its growth-repressive functions[384]. However, there is a shift in Rb phosphorylation from mitogen-dependant cyclin D-cdk4/6 complexes to mitogen-independant cyclin E-cdk2 which explains the loss of dependency on extracellular growth factors at the restriction point. The Cyclin E-cdk2 peaks at the G_1-S check point after which Cyclin E degrades to be replaced by cyclin A.

The role of p21 in urothelial carcinogenesis has not been extensively studied. We found significantly higher expression levels of p21 ($p<0.005$) in the non-muscle invasive urothelial cancer who progressed. $p21^{CIP1}$ is a member of the *Cip/Kip* polypeptide inhibitor family and was thought to be opposed to the action of cdks[385]. However, studies which revealed both $p21^{CIP1}$ and $p27^{Kip1}$ to be components of active cyclin-CDK complexes[386-388], and also that $p21^{CIP1}$ promotes the cyclin D-CDK4 complexes in-vitro[387] have necessitated a rethink about p21. Alt *et al.*[389] demonstrated that $p21^{CIP1}$ promoted nuclear accumulation of cyclin D1-CDK4. These three major biomarkers have critical roles in urothelial carcinoma progression to invasive disease through their action on the Cyclin D complexes (Figure 7.4).

Heat shock protein 27 (Hsp-27) was originally discovere as an oestrogen-modulated protein in breast cancer[390]; identified to contribute to

apoptosis[350]. It is a low-molecular weight Hsp; part of a family of highly homologous chaperone proteins, ubiquitously expressed in multiple tissues in response to environmental, physical, and chemical stresses. It has been found to be a culprit in breast[352], cervical[353] and prostate cancer[351]. Hsp-27 protects the cytoskeleton[391], regulates the serine/threonine kinase signalling molecule for cell survival[392] and other pathways implicated in the cancer cell survival and resistance[393]. This study shows a down-regulation of hsp-27 in the secondary muscle invasive urinary bladders. This suggests that overexpression of hsp-27 might have urothelial stabilising function preventing progression. Hsp-27 has been reported to have a role in the treatment of malignant disease; increased expression linked to vincristine resistance in gastric cancer[394], 5-fluorouracil resistance in colon cancer[395] and breast cancer cells which initially over- expressed Hsp-27 became sensitive to doxorubicin after modulation of endogenous Hsp-27 levels by paclitaxel[396].

Not only is Hsp-27 considered a powerful biomarker of aggressive cancers, but it has also been mooted as a therapeutic target in order to render the urothelial cells more tolerant to chemotherapy/ radiotherapy. Studies have identified areas of interest such as inhibition of tumour cell migration by blocking protein kinase C-dependent phosphorylation of Hsp-27[397] inducing mitotic arrest and enhancing apoptosis[398]; p38 MAPK/MAPK-activated protein kinase 2 (MK2) inhibitors that inhibit phosphorylation of its downstream target- Hsp-27[399]; inhibition of Hsp-27 phosphorylation at Ser^{78} and Ser^{82} by the MAPKAP kinase MK5 prevents F-actin reorganisation that

is necessary for cell migration[400]. However, further studies are needed to ascertain whether anti-hsp-27s might accelerate disease progression if it has already been initiated.

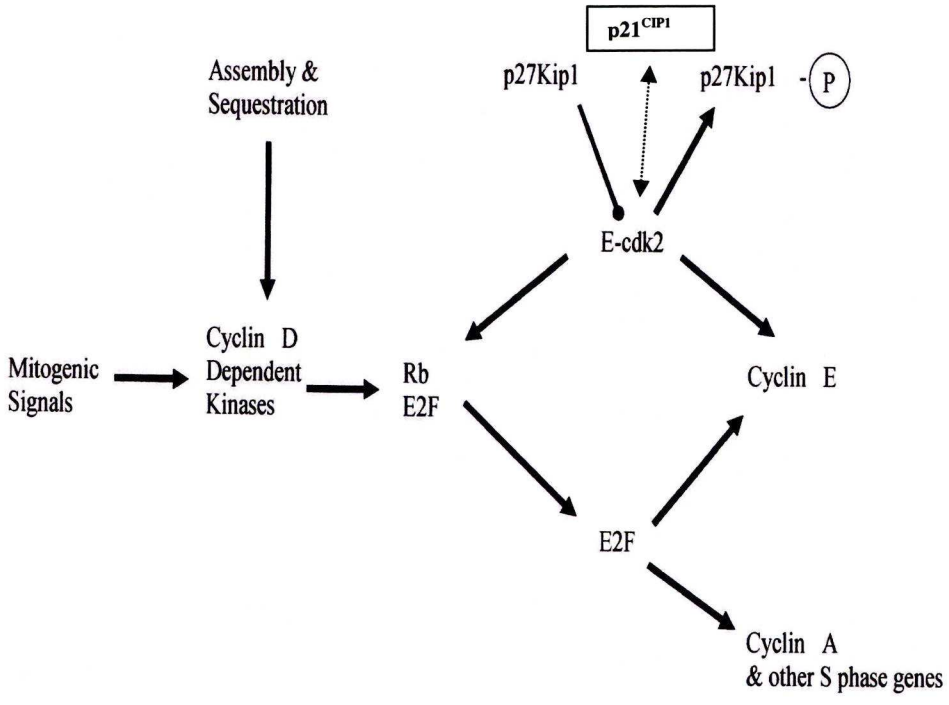


Figure 7.4: Cip/Kip proteins and cyclin cdks and Rb[383]

Chapter 8:

Conclusion

Primary bladder cancers are genotypically and phenotypically heterogenous; in common with other malignancies[401 402]. An individual phenotype is controlled by molecular signals that determine the frequency and amplitude of transcription for individual genes. A protein product encoded by one gene may cross-regulate expression of other genes. Also protein expression is variable and also could be regulated by other genes or have involvement in multiple pathways. This study has focused on identifying the molecular portraits of human urothelial carcinoma with the objective of correlating patterns of gene expression with clinical outcome. The expression of functional groups of genes have provided the basis for separating bladder tumours according to micro-anatomical location [280] so that particular gene-expression profiles characterizing each stage identify a biological phenotype, thus providing evidence for the phenotype-modulating effects of local environment.

The data obtained during this study confirms the initial hypothesis that immunophenotyping individual human urothelial neoplasms provides new predictive information on clinical behavior and outcome that is not otherwise obtainable from conventional morphology. Some of these data support, and significantly extend, the findings of several earlier studies[119 366 383 403-405]. This study also provides new information on the phenotypic events within urothelial carcinomas suggesting that the ability to predict clinical behavior or response to therapeutic intervention would be greatly improved with the benefit of biomarker profiling. Over a longer period of time, the traditional classifications may need to be altered to take into

account those patients with poor prognosis/ aggressive phenotype which would need earlier radical treatment. In analyzing the panel of tumour markers, we studied the effect of the biomarkers on an individual basis despite understanding that cellular pathways frequently overlap and are inter-woven. We felt that it would be more effective if a single or dual panel of markers could be found to improve the prognostic accuracy.

Elevation of the rate of tumour epithelial cell proliferation, using Ki-67, significantly correlates with, and predicts, individual tumour progression for some variable amount of time prior to progression becoming clinically apparent. However, this is not a simple relationship since the onset of tumour proliferation is likely to depend upon a multiplicity of different factors.

Assessment of tumour suppressor genes p53, pRb, p21 and PTEN revealed altered patterns of expression that are tumour-specific and hence contribute to determining the biological and clinical behavior of individual tumours. Of 301 cases studied, 181 (60%) revealed a probable mutation in the p53 gene. This finding corresponds with the previously-published studies of Zlotta et al.[129] and Sarkis et al.[141]. However, in many of the studied cases, expression of mutated p53, located on chromosome 17p, appear to be both gradual and heterogeneous. Typically, in these cases, the mutation appeared to originate in the basal layers of papillary tumours or premalignant epithelium and to extend through proximal layers of cells but disappearing from the cells located two or three layers from the surface of the particular epithelium. The

data indicate that, although p53 mutation is an early event in the genesis of many urothelial neoplasms, it is not an initiating event since a number of morphologically well-defined papillary lesions did not express p53 until later in the genesis of these lesions. Immunophenotypic detection of mutated p53 is potentially problematic, depending upon the particular antibody and the structural location of the specific epitope to which that antibody is directed[284]. Mutations are known to occur at many sites within the p53 gene. However, it is not known whether all mutated p53 proteins, irrespective of the location of the mutation, behave in an identically inefficient manner or whether different mutations determine distinct patterns of molecular behavior within affected cells. Detailed analysis in this thesis has been restricted to p53 as an individual marker. We found significant up-regulation of p53 expression from benign urothelium to non-muscle invasive urothelial carcinoma. The credence applied to the double hit theory is due to the the observed lower levels of p53 in muscle invasive disease.

Of the 300 cases studied, only 89 (30%) revealed loss of pRb. Rb loss was found to indicate carcinomatous de-differentiation. We were unable to find an association with stage. However, the percentage of biopsies that were both p53 and Rb mutated increased with worsening stage. Likewise, in p21 studies; this study failed to show a significant association with stage but detailed higher expression levels in muscle invasive disease. Both these markers showed significant variation between the expression levels n normal tissue in comparison with malignant biopsies. The pattern of

expression was almost reciprocal with Rb loss mirrored by p21 gain across the urothelial cancer spectrum.

	P53+Rb+ Mutated	P53+ Rb-ve	P53-ve Rb+	P53-veRb-ve Non-mutated
Dysplasia, CIS, pTa	48	32	35	13
pT1	32	8	7	4
Muscle invasive	13	6	8	1

Table 8.1: Four-group (p53 and Rb) expression patterns by stage of disease

Tumour suppressor gene PTEN, located on chromosome 10q, typically revealed a biphasic expression during evolution and progression of individual bladder cancers that is reminiscent of the expression of PKC-β in prostate cancers[406]. In non-neoplastic urothelium, irrespective of the presence of inflammation, PTEN is characteristically expressed throughout the lower layers of the epithelium. However, as in-situ neoplasia develops (pathological stage pTa), the intensity of urothelial expression diminishes and disappears. However, with subsequent stromal invasion, re-expression of PTEN occurs in the invading cells and is frequently stronger than in the corresponding non-neoplastic epithelial cells.

This study revealed a subset of non-muscle invasive urothelial carcinoma patients (despite low numbers) who behaved in a distinctly different pattern to others. These patients expressed significantly higher levels of pRb, p21 and PTEN and loss of hsp-27 suggesting a higher risk of developing

invasive muscle-invasive disease. These four biomarkers could be useful in identifying a subset of patients with non-muscle invasive urothelial carcinoma who have higher risk of progression to muscle invasive disease. These patients would be better served by earlier radical treatment than being treated with immunotherapy. It is difficult to define criteria based on these low numbers. However, it would not be easier to garner larger numbers of this specific sub-cohort as the evidential trend has been to consider early radical treatment.

Bcl-2 expression levels were highly significant within benign urothelium and loss of expression was seen early as a marker of malignant disease. Significant correlation was seen with stage. Bcl-XL expression levels were difficult to assess as the positive levels were low across the cohort. However, muscle invasive urothelial carcinoma showed significant over-expression. This suggests that Bcl-Xl over-expression could imply either a poor prognosis or resistance to urinary bladder conserving therapeutic measures

ErbB-2 loss was significant within muscle invasive urothelial carcinoma. ErbB-2 down-regulation could have a role in identifying patients with non-muscle invasive who would benefit from earlier radical treatment.

ER β appears to have a cyto-protective effective, with significant down regulation seen in malignant tissue. There was an inverse correlation of expression with tumour stage.

The phenotypic classification of urothelial tumours could be based on the four-group expression patterns of p53 and Rb. Other genes such as p21, p16, erbB-2, bcl-2 and PTEN would be used within each group for sub-group analysis.

In this study cohort, the urothelial biopsies which had no abnormality detected or had chronic inflammation in patients with a history of urothelial carcinoma were analysed as a different entity. These histopathologically normal urothelial biopsies have a distinctly separate biomarker profile in comparison with true malignancy or benign urothelium. This suggests that urothelial carcinoma is associated with field changes rather than specific focal entities. Once urothelial carcinoma has occurred, the urothelium is unstable with persistent degree of flux and needs to be monitored for life. Changes in the biomarker profile should trigger the clinician to re evaluate management plans for the patient especially in the suspected transition from pT_1 disease to muscle invasive urothelial carcinoma.

The biomarker profile of the 'no abnormality detected/ chronic inflammation with a history of urothelial carcinoma' group raise more dilemmas than answers with regards to the management and follow-up for non-muscle invasive bladder cancers. These findings imply that the use of conventional white-light cystoscopy is inadequate to diagnose these urothelial entities (not true-benign urothelium) which are not only pre-visible but also pre-histological lesions. Clinicians have realized the

inadequacies of the present protocols and tools; and have tried to improve diagnostic capabilities with the use of various technological developments such as narrow-band imaging cystoscopy[407] and fluorescent light/ photodynamic diagnostic cystoscopy[408]. However the results have been disappointing.

We need to consider a more radical follow-up program for patient with conservatively managed urothelial carcinoma. Despite there being no features of macroscopic (cystoscopic) or microscopic (histological); the presence of biomarker profiled abnormal urothelium in comparison to the benign platform suggests that it might be prudent to consider urothelial carcinoma patients for annual/ bi-annual random biopsies with a run of biomarker profiling and consequent revaluation of further management/ follow-up dependant on the favorability of the biomarker expression profile.

We are suggesting a similar evolution in managing non-muscle invasive urothelial carcinoma patients; similar to the modulated approach in conservative management of prostate cancer where annual PSA follow-up with watchful waiting has been replaced by annual/ 18-monthly prostate biopsies and an active surveillance program.

Summary

- This study has confirmed the "proof of principle" that routine immunophenotyping provides valuable pathological information important to the effective and biologically-appropriate clinical management of patients with bladder neoplasms.

- Neoplasia is confirmed and distinguished from inflammatory and other non-neoplastic conditions by loss of bcl-2 frequently accompanied by decline in expression of PTEN.

- The urothelium should be considered unstable once malignancy has been established suggesting the need for long-term probably life-long surveillance.

- Tumour aggressiveness is predicted by elevated rates of tumour cell proliferation as measured by Ki-67 assessment.

- Both papillary carcinomas and flat malignancies can be more accurately classified according to immunophenotyping expression characteristics than by morphology alone. Important parameters include p53 and pRb status that together place all bladder carcinomas into four genotypic and phenotypic groups with greater biological relevance than any unassisted morphological classification.

- Pathological staging of early malignancies, with more accurate recognition of micro-invasive disease (pT1a) rather than neoplasia

restricted to the epithelium (pTa) may be obtained by detection of hsp-27 expression.

- There appears to be a two distinct disease entities; non-muscle invasive and muscle invasive urothelial carcinoma. These two types show differing biomarker profiles which would help predict high risk non-muscle invasive disease (Mutated p53 and Rb, p21 and PTEN with loss of hsp-27).

- Three groups of genes can be established for phenotypic classification:
    1. Genes for Classification: Rb and p53
       Modifiers: PTEN
    2. Distinguishers: Malignant from benign--- erb B-2 and bcl-2
    3. Hsp-27: Not as useful in the urinary bladder but helps to identify invasion and thus makes pTa to pT_1

- The use of antibodies is time-consuming, imprecise with respect to certain antigens, labour-intensive and consequently the technique can be employed to examine expression of only a relatively few determinants. It is suggested, therefore, that molecular biological approaches are developed by which the findings of this study are extended to the analysis of the expression of a much larger range of selected genes in order to routinely expression-profile all bladder carcinomas and then obtain more accurate predictive assembly of individual cancers.

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